

Insecticidal Potential of Selected Plant Essential Oils on *Trioza erytreae* (Hemiptera: Triozidae) in Laboratory and Semi-field Conditions

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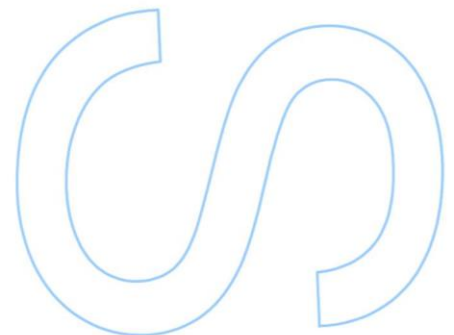
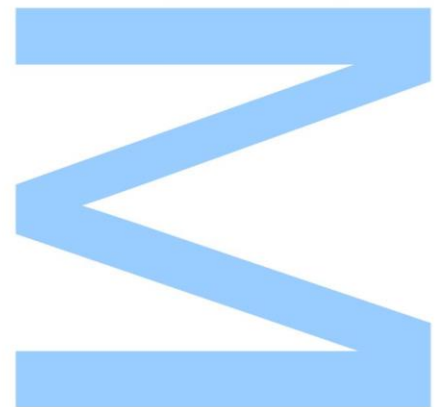
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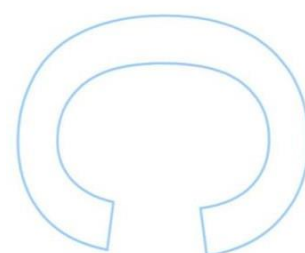
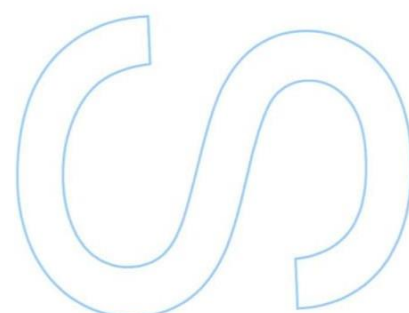
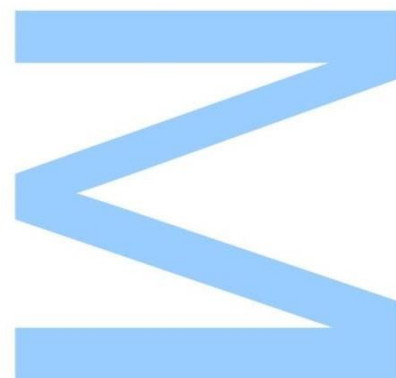




Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Resumo

O setor agrícola depende profundamente da produtividade, um fator que pode ser severamente afetado pela atividade de insetos fitófagos. Os inseticidas de síntese fornecem um controlo eficiente destas pragas em sistemas agrícolas, no entanto, os impactos negativos destes compostos sobre o meio ambiente e subsistemas ecológicos agrícolas tem levado a muitas restrições ao seu uso.

Os óleos essenciais (OEs) são metabolitos secundários sintetizados nas estruturas secretoras das plantas, atuando como mecanismo de defesa fisiológico contra insetos fitófagos. Estas substâncias podem ser potencialmente implementadas em produtos naturais para o controlo de pragas, apresentando assim uma alternativa ecológica aos inseticidas de síntese tradicionais. *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) é considerada hoje em dia uma grande ameaça à citricultura, encontrando-se atualmente em franca expansão por toda a Península Ibérica. Neste estudo, o potencial inseticida dos compostos ativos de óleos essenciais, Eugenol (99%), Pulegona (85%) e a sua combinação, foi avaliado em *T. erytreae* através da inferência da sua toxicidade (por aplicação tópica em ninfas), dos seus efeitos no comportamento de pouso e postura dos adultos, bem como dos seus efeitos ovicidas. Além disso, a fitotoxicidade destes compostos foi avaliada em *Citrus limon*, hospedeiro preferencial de *T. erytreae*. Finalmente, o tratamento que demonstrou melhores resultados foi avaliado em condições de semi-campo.

No ensaio de toxicidade, Pulegona apresentou uma maior toxicidade do que Eugenol em ninfas de *T. erytreae*, exibindo os menores valores de LD₅₀ após 24 h (0,005 µL.nymph⁻¹) e 48 h (0,003 µL.nymph⁻¹). Quanto ao LD₉₀, a combinação de ambos os compostos foi o tratamento mais tóxico, tanto ao final de 24 h (0,020 µL.nymph⁻¹), como ao final de 48 h (0,012 µL.nymph⁻¹). Além disso, a compatibilidade entre os dois compostos foi demonstrada, evidenciando um efeito sinérgico em concentrações mais elevadas após 24 h. Eugenol foi o composto mais fitotóxico em concentrações iguais ou inferiores a 3,60%, no entanto, na maior concentração testada 6,40%, a combinação dos dois óleos foi a mais fitotóxica. Respetivamente, as soluções contendo Eugenol na sua formulação apresentaram maior potencial repelente. Em condições de semi-campo, a combinação de Eugenol com Pulegona a 1,60% reduziu as populações de ninfas de *T. erytreae* em cerca de 70%.

Este estudo apresenta assim novos indícios sobre os potenciais efeitos dos OEs em *T. erytreae* assim como a aplicabilidade destes compostos em citrinos, abrindo caminho para o desenvolvimento de estratégias de controlo mais sustentáveis desta praga.

Abstract

Agricultural activity relies deeply on crop yield, a factor that can be severely affected by the activity of phytophagous insects. Insecticides can provide an efficient control of pests in agricultural systems, however, their negative impacts on the environment and on agricultural ecological subsystems has led to many regulatory restrictions on their usage.

Essential oils (EOs) are secondary metabolites synthesized in plant secretory structures and serve as a physiological defense mechanism against phytophagous insects. These substances can potentially be employed as natural pest control products, thus presenting a viable eco-friendly alternative for typical synthetic insecticides. *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) is considered nowadays a major threat for citrus production, currently expanding rapidly throughout the Iberian Peninsula. In this study, the insecticidal potential of essential oils' active compounds Eugenol (99%), Pulegone (85%) and their combination was assessed by determining their toxicity (through topical application on *T. erytreae* nymphs), their effects on adults' settling behavior and oviposition, as well as their ovicidal effects. Furthermore, the phytotoxicity of these compounds was evaluated on *Citrus limon*, *T. erytreae* preferred host. Lastly, the compound with the best results was evaluated in semi-field conditions.

On the toxicity bioassay, Pulegone was more toxic to *T. erytreae* nymphs than Eugenol, showing the lowest LD₅₀ values both after 24 h (0.005 µL.nymph⁻¹) and 48 h (0.003 µL.nymph⁻¹) whereas for the LD₉₀, the combination of both oils was the most toxic both after 24 h (0.020 µL.nymph⁻¹) and 48 h (0.012 µL.nymph⁻¹). Furthermore, compatibility between both active compounds was demonstrated, with evidence of a synergistic effect on higher concentrations after 24 h. Eugenol presented the highest phytotoxic effect on concentrations equal or lower than 3.60% whereas for the highest concentration, the combination of both oils was the most phytotoxic. Respectively, Eugenol-containing solutions also presented a higher repellent potential. In semi-field conditions, the joint action of Eugenol and Pulegone at 1.60%, decreased by 70% the populations of *T. erytreae* nymphs.

This study presents new insights on the potential effects of EOs on *T. erytreae* and their applicability in citrus crops, thus making an entry way for the development of more sustainable control strategies for this pest control.

Keywords: African citrus psyllid, *Citrus limon*, Crop protection, Integrated Pest Management, Monoterpene, Oviposition, Phytotoxicity, Repellent, Synergism, Topical toxicity.

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Abbreviations

BD	Bud/shoot Death
CE	Chlorophyll Evolution
ECE	Effective Chlorophyll Evolution
EO	Essential Oil
E	Eugenol
EP	Eugenol and Pulegone combined solution
GN	Greenhouse Nymphs
HLB	Huanglongbing
HR	Hatch Ratio
IDT	Instar Development Time
IN	Insectary room Nymphs
IPM	Integrated Pest Management
IR	Interaction Ratio
LDth	Leaf Death
LD	Lethal Dose
MR	Mortality Rate
N1-5	Nymphal stage 1-5
NB	Necrotic Blots
NS	Necrotic Spots
PD	Petiole Discoloration
PI	Phytotoxicity Index
P	Pulegone
RC	Relative Chlorophyll
SD	Stem Discoloration
VC	Visual Classification

1. Introduction

Agricultural practices and their economic viability as a whole are highly reliant on crop yields, which can be affected by several factors, from which phytophagous insects are especially relevant. These pests influence on crop losses can become a little difficult to determine due to all the interactions present in the agricultural environment (Grzywacz et al., 2014), however, estimates establish that an average of 35-40% of food crop losses are related to the activity of phytophagous insects (Pavela, 2016).

Undoubtedly, insecticides have played a fundamental position in assisting agricultural production until this day, but their negative impacts on the environment and even on human health have led to many regulatory restrictions on their usage. Additionally, excessive usage or misuse of insecticides may also have detrimental impacts on agriculture itself due to its contribution to the contamination of water, soil and air, while also affecting the functional biodiversity of the agricultural system by alteration of the balance in its ecosystem and eliminating or reducing non-target beneficial organisms and insects, including not only pest predators and parasitoids, but also pollinating and agronomic interest insects such as bees (Stoytcheva, 2011)

Meanwhile, knowledge on plant-pest-predator interactions led to the development and study of the potential usage of plants secondary metabolites for pest control, with numerous new products appearing in the market (Miresmailli & Isman, 2014). Plants are sessile organisms, exposed daily to a wide array of biotic and abiotic stresses as they battle and compete with other organisms for available resources. This constant struggle leads to the inevitable pursuit of competitive advantages, allowing the plant, and more broadly the species, to thrive in its habitat (Verdeguer et al., 2020). The search for this competitive advantage ends up resulting in the modification of its physiology, allowing the plant to respond and/or better prepare itself for such threats (Aroca, 2012). Certain plants have evolved to develop a broad spectrum of physiological defences against a wide variety of insects, an ability that can be reused for the production of natural pest control products (Pavela, 2016).

Essential oils (EOs) are synthesized in plant secretory structures and present a preponderant role in plants' direct and indirect defenses against herbivores and pathogenic organisms (Regnault-Roger et al., 2012). These oils composition is species dependent and comprises a mosaic complex with a variable number of polar and non-polar molecules in different concentrations. Frequently, 2 to 3 components are

predominant in their concentration (20-90%) (Falleh et al., 2020) often determining each oil's bioactivity (Raut & Karuppayil, 2014).

Usage of EOs and aromatic plants for medicinal and/or ceremonial purposes goes back several millennia but their usage as a reliable pest control only reached interest among the scientific community in the last two decades (Isman & Grieneisen, 2014; Isman & Tak, 2017). Although the approval process can be an obstacle to the entry of new products to control crop's pests and diseases in the market (Isman & Tak, 2017), new products formulated with essential oils are starting to appear all over the world, including Europe (Raveau et al., 2020). Essential oils efficacy against insects is well known and its fumigant, contact and repel properties are well documented against a vast array of specific agricultural pests (Isman, 2000), including several arthropods from the Hemiptera order of species (Mann et al., 2012; Rizvi et al., 2018; Silva et al., 2016; Tian et al., 2015; Volpe et al., 2016).

Trioza erytreae Del Guercio (Hemiptera: Triozidae) is nowadays considered one of the major threats to global citrus production (Cocuzza et al., 2017). This small psyllid is the vector of a bacteria named *Candidatus liberibacter africanus*, one of the three causative bacteria of Huanglongbing (HLB), a devastating citrus disease (Bové, 2006; Yang et al., 2016). Native from Africa, *T. erytreae* is known to be present in the Iberian Peninsula since 2014 and distributed wildly through the coastal region of Portugal during the following years (Cocuzza et al., 2017; Paiva et al., 2020). This region has served as the introduction of this pest to continental Europe, and great awareness has since been raised for its control, with quarantine measures taking place throughout most of the coastal regions of Portugal and Spain (DGAV, 2021; DGSPA, 2021). Since this species has already shown great adaptability to a variety of ecological conditions, its continuous spread to other regions of Europe seems very apparent (Cocuzza et al., 2017). While HLB has not yet been identified in Europe, containment of the spread of its vector is imperative, especially in the Mediterranean Basin, the world's second-largest citrus-growing region and the main citrus commercial center (Dionisio et al., 2021; Urbaneja-Bernat et al., 2020). Control of *T. erytreae* is nowadays assured by quarantine measures, cultural practices, farm sanitation with insecticides and the controlled release of its native parasitoid *Tamarixia dryi* (Hymenoptera: Eulophidae) (DGAV, 2021; Van den Berg et al., 1991). Although the usage of insecticides can show good results in controlling local populations, their widely known negative effects may limit the effective control of its expansion (Antwi-Agyakwa et al., 2019; Belasque Jr et al., 2010; Kilalo et al., 2009). Biological control by the release of *T. dryi* is also showing great results but one should

account for its unknown efficacy on continental land, especially when the equilibrium between pest-parasitoid populations is established.

The efficacy of EOs in *T. erytreae* related species (Mann et al., 2012; Rizvi et al., 2018; Silva et al., 2016; Tian et al., 2015; Volpe et al., 2016) shines promise on the usage of these compounds as an alternative or a complement to all the current used control methods. However, the effectiveness of these compounds on *T. erytreae* has not yet been properly tackled, with only recent literature showing that some compounds may have certain effects in this specie (Antwi-Agyakwa et al., 2019; Dionisio et al., 2021), further validating the need to expand the knowledge on this topic.

Given this gap in knowledge on the efficacy of such compounds on *T. erytreae*, some hypotheses are thus raised: i) These compounds are effective in controlling *T. erytreae* and affect this psyllid various life forms; ii) There are no implications related to plant phytotoxicity when applying these compounds on *Citrus limon* (*T. erytreae* preferred host), thus, they can be applied directly without concerns related to plant health; iii) The usage of the combination of these compounds is beneficial in the context of crop protection; iv) These compounds can maintain their effects in field conditions and are thus viable alternatives in a practical context.

In this work, to determine the insecticidal potential of EOs' active compounds on *T. erytreae*, Eugenol, Pulegone and their combination are to be evaluated for their:

- i) Direct contact toxicity;
- ii) Residual effect repellency;
- iii) Ovicidal effects.

To verify their applicability in citrus crops, an appraisal of the phytotoxic effects of these compounds on *C. limon*, is also to be assessed. A semi-field assay was also implemented to better understand the effectiveness of these compounds in reducing *T. erytreae* populations on *C. limon* in a practical context, as well as comprehend the influence of altering application dosages of the studied treatments.

2. State of art

2.1. Essential oils

2.1.1. The use of insecticides in agriculture - Need for alternatives

The influence of chemical pesticides and other control agents on non-target organisms, and specifically biological control agents, has been a matter of great awareness due to the integration of these agents in Integrated Pest Management practices. Adding to the direct mortality caused by the application of synthetic insecticides, physiological sublethal effects on the development of beneficial arthropods can also occur at multiple levels with reported perturbations of the foraging patterns on parasitoids, interference on their feeding behavior and prevalence of malformations on subsequent generations (Desneux et al., 2007). In a society increasingly sensitive to the impacts of food production, namely those related to the effects of the use of phytopharmaceuticals on the environment and human health, the search for alternatives to synthetic products has been a central theme of debate among the scientific community.

Currently, there are several options on the market capable of ensuring efficient control of pests and disease vectors. Many of these alternatives were developed by applying knowledge from the areas of genetic engineering and genetic improvement that, among other topics, investigate plant-pest-predator interactions (Miresmailli & Isman, 2014). Certain plants have evolved to develop a broad spectrum of physiological defenses against a wide variety of insects, a capacity that can be reused for the production of natural pest control products (Pavela, 2016).

2.1.2. Origin and current scientific overview

EOs are molecules synthesized by secondary metabolic pathways, which play a preponderant role in the plant's direct and indirect defence against herbivores and pathogenic organisms. Present in secretory structures such as glandular trichomes, secretory cavities and resinous ducts, EOs can be extracted from many different parts of the plant - shoots, floral petals, stem rhytidome, leaves, stamens, seeds, roots, resins and fruit peels (Regnault-Roger et al., 2012) – however, its biological effects may be dependant on the plant part from wich it was exctracted (Raut & Karuppayil, 2014).

EOs are mainly extracted utilizing low or high pressure distillation using boiling water or hot steam, but can also be extracted using liquid carbon dioxide or microwaves, albeit less commonly (Bakkali et al., 2008). Quality, quantity and chemical profile of the substances are dependent on the extraction method employed, as well as the source-plants vegetative life cycle and edaphic and climate conditions on which it was produced (Angioni et al., 2006; Masotti et al., 2003). It is estimated that aromatic plants and their oils have been used for at least 6500 years in Egypt, 4500 years in China, and 3000 years in India but it is of current belief that their usage may be even older. In their early days, these oils were widely used for medicinal and/or ceremonial purposes, but, according to popular evidence, they have been applied as insect repellents for several hundred years (Isman & Tak, 2017).

Despite the demonstrated biocidal potential, scientific research on the repellent and toxic effects of EOs is much more recent, with the first publication on the topic appearing in 1947 (Sharma, 1974). Although the preliminary results were promising, the interest in these substances' bioactive properties with insecticidal effects remained relatively low until the end of the 20th century. It was from the year 2000 onwards that the scientific community's interest in the topic gained greater expression. In 2012, the proportion of articles published on EOs represented more than 1/4 of all articles published on insecticides (Isman & Grieneisen, 2014). The avalanche of information and knowledge obtained in the last decade made possible the formulation of new commercial insecticides based on EOs.

2.1.3. Composition and mechanisms of action

EOs are characterized by a strong odour with its intensity dependent on the chemical composition and concentration of the different components. The composition of these oils comprises a mosaic complex with a variable number of polar and non-polar molecules in different concentrations. Frequently, 2 to 3 components are predominant in their concentration (20-90%) (Falleh et al., 2020), which often determine the bioactivity of the oil (Raut & Karuppayil, 2014). Evidence suggests that many secondary constituents of an EO play a role in the fragrance, texture, density and cell penetration properties of the oil (Cal, 2006), nonetheless, the major compounds within the oil seem to reflect quite well the biological properties of the extraction source EO (Bakkali et al., 2008).

Since plant EOs often comprise a great number of constituents, they can be expected to have various targets of action (Tak et al., 2016). As typical lipophiles, EOs have the ability to break the cell wall and cytoplasmatic membrane, which not only may lead to cell lysis, but may also facilitate the entrance of other toxic compounds into the cytoplasm (Pavela & Benelli, 2016). EOs constituents also target detoxifying enzymes such as the P450 cytochromes (Regnault-Roger et al., 2012) and specific receptors in the insect nervous system such as γ -aminobutyric acid (GABA) (Enan, 2005; Priestley et al., 2003), octopamine (Enan, 2001; Enan, 2005), tyramine, or acetylcholinesterase (AChE) (Mills et al., 2004; Regnault-Roger et al., 1993; Tak & Isman, 2015). These modes of action are akin to the activity of many insecticides, that often target the insects' nervous systems (Matsumura, 1975).

Due to these different mechanisms of action, these compounds can thus interact often exhibiting synergistic, additive and/or antagonistic effects (Gaire, Scharf, et al., 2020). These effects may occur not only between the different components present within the oil, but also in mixtures of monoterpenoid compounds (Ma et al., 2014), essential oils (Zhang et al., 2016), and their combination with synthetic insecticides (Dionisio et al., 2021; Pennetier et al., 2005).

An additive effect is noted when the combined effect of two compounds is equal to the sum of their individual effect (Bassolé & Juliani, 2012). Synergic interaction between two compounds understands that their effect is greater when combined, then their sum when applied as separate products. Such interaction is based on the rationale that these compounds have multiple modes of action, hence, the sum of their bioactivities produces a greater toxic effect. Moreover, the antagonistic interaction is observed when the combined effect of a mixture of compounds is lower than the individual effect of each of those compounds by themselves (Ntalli et al., 2011). For the prospect integration of EOs in pest management practices, the study of these interactions is of great relevance. From these, the potential synergistic effect of these interactions is especially pertinent, as it could allow the formulation of more effective products with lower dosages, all while reducing the risk of resistance development (Pavela, 2015; Tak et al., 2016).

As for the motifs behind the synergistic interactions, these can still be unclear. However, based on pharmacological studies, Wagner and Ulrich-Merzenich (2009) specified four major possible mechanisms:

- i) *“synergistic multi-target effects”*, meaning that the constituents within the plant extract affect multiple targets, hence cooperate in augmenting the toxicity in a synergistic way;
- ii) *“pharmacokinetic or physicochemical effects based on improved solubility and resorption rate”*: while a specific constituent may not exert toxicity by itself, it increases the resorption rate of other toxic compounds in the mixture, enhancing their bioavailability and thus augmenting their toxicity;
- iii) *“interactions of agents with resistance mechanisms”*, meaning that a compound may have the ability to suppress the organism’s defense mechanisms to a certain toxic compound, increasing its toxicity;
- iv) *“respective elimination or neutralization of adverse effects”*: an otherwise neutral agent in the mixture augments the effectivity of the extract by neutralizing or destroying another compound that is reducing its toxicity.

On insects, EOs, and more specifically terpenoids, can have a large range of effects classified according to the induced response, that is, behavioural (repellent, attraction and deterrence) and physiological (toxicity and inhibition of growth and development) (Dancewicz et al., 2008; Tripathi et al., 2009). Given the complex composition of these oils, both behavioural and physiological effects can appear simultaneously in the same individual. The simultaneous occurrence or overlapping of these effects can occur due to two factors (Isman & Tak, 2017):

- i) in their volatile form, they are intercepted by the insect in its chemosensory organs and absorbed by the tegument, producing behavioural and/or physiological effects;
- ii) its influence on the production of octopamine, which can affect numerous physiological and behavioural systems in insects.

2.1.4. Essential oils as low-risk compounds

EOs can be used as an alternative to traditional pesticides, mimicking their effects on target organisms while avoiding toxicity to non-target species, thus being more selective towards pest targets (Copping & Duke, 2007).

Despite the barriers in their characterization, one of the most attractive points in the application of EOs is the fact that they are recognized as low-risk products: their toxicity in mammals is low and, additionally, many of their effects have also been extensively tested, both experimentally and clinically, due to its use in medicinal products.

Most EOs have a LD₅₀ value in guinea pigs between 2000 and 5000 mg.kg⁻¹. Isolated constituents show similar toxicities, with very few having a LD₅₀ lower than 2000 mg.kg⁻¹ (Regnault-Roger et al., 2012). As a comparison, the compound imidacloprid, commonly used as an insecticide until 2019, has a LD₅₀ per ingestion in guinea pigs of 131 mg.kg⁻¹ (Kidd & James, 1991), which is markedly lower and therefore much more toxic. In terms of ecotoxicology, EOs are safe, however, their use must be analysed on a case-by-case basis and in a responsible manner. Its constituents are biodegradable, with half-life degradation times ranging between 30 hours and 18 days, with low persistence in the environment, without the occurrence of bioaccumulation or bioaugmentation, contrary to what happens with some synthetic insecticides (Misra & Pavlostathis, 1997; Peterson & Ems-Wilson, 2003).

Despite their general safety, when using EOs one should consider their possible allelopathic and phytotoxic effects. Due to a typical short residual activity and restricted entry intervals <12 h, to maintain the insecticidal effects repeated applications may be required and may result in phytotoxicity (Miresmailli & Isman, 2006). In fruit plants, phytotoxicity may be evaluated by observing discoloration of the whole leaf lamina (chlorosis, whitening and local leaf discoloration); discoloration of current year's shoots (discoloration, number and appearance of lenticels) and necrosis of leaves (edges, along the veins and the whole leaf lamina) (EPPO, 2014). Other symptoms on the plant are also indicative of a phytotoxic effect such as leaf distortion, stunting of growth, leaf and roots burn and even excessive growth stimulation and elimination and distortion of fruit or flowers (Ibrahim et al., 2001). Photosynthetic parameters such as the decrease of chlorophyll content can also provide an insight on the plants general health as they are recognized as reliable indicators of many biotic stresses (Huang et al., 1997). It is advised that phytotoxic evaluations should be conducted on young plants in pots, as these are often more sensitive, and thus possible manifestations of phytotoxicity could be monitored right from the initial tests (Ikbal & Pavela, 2019).

Phytotoxic activity of EOs is attributed to the activity of the various compounds within the oil and their interactions - often synergistic - including enzyme inhibition, chlorophyll degradation, electrolyte leakage and membrane damage (Kimbaris et al., 2017). Phytotoxicity and the severity of the plant injury may be dependent on the concentration and dosages applied, plant species, and plant organs exposed during the application, the physiological state and product formulation (Cloyd et al., 2009; Werrie et al., 2020). Phytotoxic effects can be observed for several oils and seem to be both species and dosage dependant. Some examples are the oregano (*Origanum vulgare*) and *Cymbopogon citrates* oils, which can lead to growth inhibition of certain maize and tomato cultivars (Dudai et al., 1999). Allelopathic effects were also observed in maize, in the inhibition of root development, by the application of oil from *Tagetes minuta* and *Schinus areira* (Scrivanti et al., 2003). Suitably, Carvone (abundant in the oils of *Carum carvi*) can be used in stored potato by keeping it from resprouting (Reust, 2000).

Since EOs are generally regarded as a safer alternative to conventional chemical pesticides, the phytotoxic effects of these compounds on crops are often not accounted for. The search, study and catalogue of specific oil-crop interactions, as well as the causes behind their observable phytotoxic effects may thus present an interesting study opportunity, aiding the decision-making on formulating plant-based insecticides.

2.1.5. Essential oils as biopesticides: Current overview of EO commercialization

The use of EOs in agriculture, and their application methods, differs in developing and developed countries. While in developing countries, insecticidal effects produced by aromatics plants soon started aiding the agricultural practice, especially in the control of pests in stored crops, the lack of resources to implement a regulatory framework around these practices can lead to dangers arising from their incorrect handling. Additionally, the great diversity of the autochthonous flora found in these countries potentiates the study and development of new and more effective plant-based products, however, the scientific research needed to obtain these data is often compromised by lack of funding (Regnault-Roger et al., 2012).

On the other hand, in developed countries several oils are already included in the formulations of commercially registered products. Among these, the most common are the oils of garlic (*Allium sativum*), clove (*Syzygium aromaticum*), cedar (*Juniperus*

virginiana), peppermint (*Mentha piperita*) and rosemary (*Rosmarinus officinalis*) (Regnault-Roger et al., 2012).

Recent years have seen an increase in interest in EOs, as noted by the increase in scientific publications related to their biocidal properties. Despite the growing scientific literature that proves their effects, there are still few commercialized insecticides that use EOs in their formulation, and even fewer those that have been successful in the market. In most countries, processes related to the regulation of biopesticides and biological control agents are still subject to the requirements applied to the production of synthetic chemical phytopharmaceuticals, even though the reduced danger of OEs has already been demonstrated (Villaverde et al., 2014). The complexity and slowness of regulating a new product based on OEs can often only be supported, with financial viability, by well-established companies in the market, creating a barrier to entry for smaller and specialized entities that try to obtain approval for new products that will compete in the market of conventional phytopharmaceuticals (Isman & Tak, 2017). Currently, efforts are being made to alleviate restrictions in the registration processes of new products considered as low-risk, that is, products that do not show toxicity to non-target organisms and with a low persistence in the soil (Villaverde et al., 2014).

The number of biological control agents and more specifically EO-based products remains quite low in Europe, especially when compared to the United States market (Olson, 2015). Thanks to a simplified registration process in the USA, a product based on certain EOs, namely those that are used in the food and beverage industry is exempt from approval and can continue its development process. Thus, this opportunity allowed the formulation of products with fungicidal, herbicide or insecticide effects, using thyme (*Thymus vulgaris*), clove and rosemary EOs as main compounds (Kola, 2011; Koul et al., 2008).

More recently, in Europe, an increasing number of OEs have been approved for use in agriculture. Therefore, always considering the purpose of use, several plants and their oils have already been authorized, namely *Mentha arvensis* and *Mentha spicata*, *Juniperus mexicana*, *Citrus x sinensis*, *Persicaria odorata*, *Piper nigrum*, *Canarium commune*, *Cinnamomum zeylanicum*, *Boswellia carterii*, *Cymbopogon flexuosus*, *Litsea cubeba*, *Artemisia alba*, *Cistus ladaniferus*, *Copaifera tree*, *Ferula galbaniflua*, *Citrus aurantium* and *Schinus terebinthifolius* (Raveau et al., 2020). As a result of this increase in authorized substances, approved commercial products have already started to appear in Europe, such as: *BIOXED*[®], which uses Eugenol (active substance from clove oil) as a fungicide or bactericide agent, targeted at pathogens present in apples and pears

storage; *BIOX-M*[®], which uses peppermint oil as a potato growth regulator; *LIMOCIDE-OROCIDE-PREV-AM*[®] and *ESSEN'CIEL*[®] which use orange EO to control whitefly, potato beetle and tobacco thrips (Raveau et al., 2020).

In Portugal, EOs are recognized as a biopesticide and follow the regulatory trends in the European Union, although subject to specific authorization for their use in national territory. The authorization of each substance is analysed individually according to its action: fungicide or insecticide. Of all the active substances approved by the European Union in Portugal, several EOs and/or their active substances are already authorized as fungicide, such as Eugenol, geraniol (found in EOs of *Cymbopogon martinii*) and thymol (*Thymus* spp.). As insecticides, the use of orange oil, rapeseed oil, azadirachtin, pyrethrins, garlic extract and clove oil are also authorized (Oliveira, 2020).

In recent years there has been a growing interest in the production of insecticides with EOs and, although this interest has not yet been reflected in increased availability of these products on the market, the next few years are promising: i) society continues to show a trend of preference for products of biological origin, an expanding market that may show increased interest in the use of EOs; ii) international efforts are being made to simplify the authorization and registration process for low-risk products, which will allow for a greater flow in the EO-based phytopharmaceutical market; iii) due to the growth in trade between countries, combined with climate change, the introduction and establishment of exotic pests have been increasingly common, exacerbating the need for continuous search for new pest control techniques. Despite the low levels of extraction yield of these oils, which significantly increase their market price, the genetic improvement of cultivars with interesting extraction rates may result in a yield increase, reducing production costs. The advantages of using these oils are already known and scientific progress in the area has proven their potential, allowing government agencies greater flexibility in their regulation. New social trends and globalization create opportunities for the development of new products for tackling and controlling pests, thus creating the necessary space for the emergence of new products based on EOs in the most varied markets.

2.1.6. Eugenol

Eugenol (C₁₀H₁₂O₂) is a phenolic aromatic substance, usually found in the form of a yellowish oily liquid, belonging to the allyl-benzene class of phenylpropanoids. It was first isolated in 1929 as a volatile compound and is present mainly in EOs of *S.*

aromaticum buds (also known as clove) and leaves (45-90%); cinnamon leaves (*C. zeylanicum*, *Cinnamomum cassia*, and *Cinnamomum verum*); while it has also been reported at varying ratios in the EOs of other plant species (Marchese et al., 2017). Eugenol can also be synthesized through the allylation of guaiacol with allyl chloride and be biotransformed via the involvement of various microorganisms (Mishra et al., 2013).

Clove oil is known for its low oral and dermal toxicity and relatively few environmental effects (Copping & Duke, 2007) and Eugenol is recognized for both its insecticidal and herbicidal activities. As a botanical insecticide, the usage of Eugenol is well-known and is recommended for use against a wide range of insects including aphids, armyworms, beetles, cutworms, grasshoppers, loopers, mites and weevils in fruit and vegetable crops and can act as a deterrent for many species (Copping & Duke, 2007).

EO of *S. aromaticum* and Eugenol is a well-known botanical insecticide (Copping & Duke, 2007), which is already homologated for usage in biopesticides in Portugal (Oliveira, 2020).

This compound has shown to be effective in direct contact assays against hemipterans such as *Drosicha mangiferae* (Gaire et al., 2019; Ghafoor et al., 2019), *Cacopsylla chinensis* (Tian et al., 2015), *Triatoma infestans* (Moretti et al., 2013) and *Aphis craccivora* (Tewary et al., 2006) and fumigant activity was also observed against *Bemisia tabaci* (de Carvalho Ribeiro et al., 2010) and *Trialeurodes vaporariorum* (Choi et al., 2003). Repellency is another widely known effect associated with Eugenol (Dardouri et al., 2019; Diaz-Montano & Trumble, 2013; Hall et al., 2018; Moretti et al., 2013; Obeng-Ofori & Reichmuth, 1997; Pérez-Ramirez et al., 2014; Zhang et al., 2014), with observed deterrence and ovicidal effects (de Andrade et al., 2013; Dias et al., 2019). Eugenol as also been a great focus of attention due to its ability to hyperactivate insects metabolism in lower dosages (Reynoso et al., 2018), a property may be responsible for its high synergistic potential (Enan, 2001; Gaire, Scharf, et al., 2020; Moretti et al., 2013).

Even though Eugenol is also known for its herbicidal activities and is regarded as highly phytotoxic, literature may be a bit divisive on the topic. As an herbicide, clove oil is a contact, non-selective foliar herbicide that will only control above-ground, green vegetation. It causes rapid loss of cellular membrane integrity and does not translocate (Copping & Duke, 2007). Phytotoxicity was observed for some monoterpenoids on corn roots and leaves with L-Carvone and Eugenol as the most phytotoxic, whereas Pulegone was the safest with no observable phytotoxic effects (Lee et al., 1997). Several EO-based commercial products containing Eugenol or clove oil were noted to be highly phytotoxic (Cloyd et al., 2009). Hall et al. (2018) reported extensive necrosis of mature

citrus leaves as well as shoots of young plants when treated with concentrations over 5%. Contrarily, in unpublished data referenced by Tian et al. (2015) no phytotoxicity was observed when clove essential oil was applied to pear trees at 10000 ppm.

2.1.7. Pulegone

Pulegone is a monoterpene ketone found mainly in EOs extracted from plant species belonging to Lamiaceae family. Pulegone was first isolated from the EO of *Mentha pulegium* (also known as pennyroyal), hence its name (Božović & Ragno, 2017). The amount of Pulegone present in the various oils can vary depending on the origin of the plant, yearly weather conditions, harvest date, plant age, fertilization, location and planting time, but is found in consistently higher concentrations (70 - 97%) in the oils of *M. pulegium* (Farley & Howland, 1980; Franzios et al., 1997; Kokkini et al., 2002; Weglarz & Zalecki, 1985).

Although widely used as an insect control agent, ingestion of Pulegone can lead into the presence of toxic metabolites including methofuran, a protein-binding agent that can cause toxic acute effects (Tripathi et al., 2009). Acute oral toxicity of Pulegone (LD₅₀=470 mg.kg⁻¹) in mice was reported by the European Scientific committee on Food Ingestion (Rossi et al., 2012); it can be potentially hazardous to the lung and liver of mice (Gordon et al., 1982); reduction in cell viability was observed by Sánchez-Borzzone et al. (2017) when applying Pulegone at high concentrations; toxic effects in humans were also associated with the ingestion of pennyroyal EO (Anderson et al., 1996). However, this compound is given the Generally Recognized as Safe (GRAS) status by the United States Food and Drug Administration (USFDA) and is approved by FDA for food use (21 CFR 172.515). It is also included by the Council of Europe in the list of artificial flavoring substances that may be added temporarily to foodstuffs without hazard to public health (Borau et al., 2020).

Pulegone, alongside menthone (when present) is responsible for many of the active properties of the *M. pulegium* oil, but is regarded as the most effective compound against arthropods (Domingues & Santos, 2019). Pulegone and *M. pulegium* containing high concentrations of Pulegone (>75%) have shown its effectiveness on hemipterans in direct contact assays against *Planococcus ficus* (Borau et al., 2020), *Metcalfa pruinosa* (Kim et al., 2013), and numerous species of aphids (Ikbal & Pavela, 2019). Fumigant activity has also been reported on *P. ficus* (Peschiutta et al., 2017) as well as repellency on *Rhopalosiphum padi* (Pascual-Villalobos et al., 2017). Synergistic and antagonistic

effects have also been observed for Pulegone, and seems to be highly reliant on the compatibility of this compound with the others in the mixture (Domingues & Santos, 2019).

Phytotoxicity of *M. pulegium* EO can also be divisive, as contradictory information can be found. While this oil has shown to have potential on weed control, with greater effect than that of commercial herbicides (Hanana et al., 2017), in the small number of studies that investigated its phytotoxicity in crops, *M. pulegium* EO, and especially Pulegone, has been shown to be safe for usage, with moderate to even zero observed phytotoxicity, and effectively lower than that of Eugenol (Borau et al., 2020; Kimbaris et al., 2017; Lee et al., 1997; Papadimitriou et al., 2019; Santana et al., 2014; Topuz et al., 2018; Zandi-Sohani, 2011; Zandi-Sohani & Ramezani, 2015).

2.1.8. EOs for *T. erytreae* and related insects' control, and field application methods

Regarding the usage of EOs as a combat strategy, several assays have been performed against *T. erytreae* taxonomically related insects, showing promising results concerning the usage of EOs as an insecticidal agent.

A recent study evaluated the potential effects of plant volatiles in *T. erytreae* adults. The experiments were conducted in laboratory conditions to understand how *T. erytreae* adults interact with the volatiles of one of its preferred host plants *Citrus jambhiri*, commonly known as rough lemon. The results have shown that *T. erytreae* flight behavior is influenced by plant volatiles, shining promise on the integration of other EOs active compounds in the control of its populations in citrus orchards (Antwi-Agyakwa et al., 2019). The same author also explored the intercropping potential of non-host plants with *Citrus limon* where it was observed that some volatiles decreased the attraction of *T. erytreae* adults (Antwi-Agyakwa et al., 2021). A result that agreed with the behavior observed on the related species *Diaphorina citri* (Gottwald et al., 2010; Mann et al., 2011; Zaka et al., 2010). A clear preference for lemon volatiles than those from bitter orange was also observed by Benhadi-Marín et al. (2021).

D. citri Kuwayama (Hemiptera: Psyllidae), also known as the Asian citrus psyllid is along with *T. erytreae* one of the known vectors of HLB. Since the arrival of HLB in Florida via this insect in 2005 (Hodges & Morse, 2009), this small psyllid has been the subject of various studies regarding the potential application of EOs. As such it is known that *D. citri* is repelled by *Allium tuberosum* EO (Mann et al., 2011) and *Psidium guajava* oil

extracts (Silva et al., 2016); is affected by *Artemisia absinthium* EO, with acute toxicity observed both in topical and residual contact applications not only for the oil as a whole but also by its main active ingredients carvacrol (*O. vulgare* EO), (-)- α -bisabolol (*Matricaria recutita* EO) and chamazulene (*Matricaria chamomilla* EO) (Rizvi et al., 2018); Dillapiol, the active compound of *Piper aduncum* has also shown exhibit great toxicity on *D. citri* nymphs and adults at 0.75% and 1% dilutions (Volpe et al., 2016).

Recently, the effectiveness of pine oil was assessed as an organic-based adjuvant in pesticide-based treatments against *T. erytreae*. This study was conducted both in laboratory and semi-field conditions and assessed the potential usage of this oil in association with known effective pesticides, deducing the mortality of all components tested both on the pest and its nymphal parasitoid, *T. dryi*. The results were conclusive: although this oil by itself did not show great toxicity, its combination with several insecticides provided higher mortality on the pest, while reducing its effects on *T. dryi*, when compared with the application of the same insecticides individually (Dionisio et al., 2021).

Tian et al. (2015) has also demonstrated the potential usage of *S. aromaticum* as a viable EO for the control of *C. chinensis* (Yang and Li) (Hemiptera: Psyllidae), where the author observed the strong contact toxicity of Eugenol against both nymphs and adults.

In fact, the efficacy of these compounds has also been demonstrated in field and semi-field conditions through spray application in both *T. erytreae* and *C. chinensis*, where the obtained results contrast favorably with the ones observed in the laboratory assays (Dionisio et al., 2021; Tian et al., 2015). The spray application of pesticides is the go-to application procedure for crop protection due to its simplicity, cost-effective nature and applicability ease. However, this can be a wasteful process, often translating to an inefficient control of the pest, all while affecting non-target organisms and the environment, as well as having an impact on production costs (Cunha et al., 2012; Salyani et al., 2007). Thus, the correct adjustment of the spray application parameters remains a topic of great relevance on crop protection studies, where an intricate relationship between the efficacy of spraying applications and the spray coverage of the target has been determined numerous times (Cunha et al., 2012). Water-sensitive papers are stripes of paper in varying sizes, with a yellow surface that turns dark blue when in contact with an aqueous substance. The change in color is a result of the changing pH in the surface of the paper when water interacts with the bromophenol-blue dye. This reaction thus allows the inference of droplet size and coverage in the target surface (Marcal, 2018). Water-sensitive papers are thus useful as an indicator related to crop spray quality provided that their limitations are considered (Cunha et al., 2012).

The importance of *T. erytreae* as an emerging and rapidly expanding pest, whom no effective control methods have yet been obtained, and the shown knowledge on the effects of EOs in *T. erytreae* and taxonomically related species, thus entails the necessity of further assessing the potential usage of these compounds as a viable alternative method to its control, both by their application in laboratory as well as in field conditions.

2.2. *Trioza erytreae*

2.2.1. *T. erytreae* as a vector of Huanglongbing

Trioza erytreae Del Guercio (Hemiptera: Triozidae), also known as African citrus psyllid, is a member of the Triozidae family and constitutes nowadays a serious threat to global citrus production (Cocuzza et al., 2017). *T. erytreae* is a vector of the bacteria *Candidatus liberibacter africanus*, one of the three causal agents – *Candidatus Liberibacter africanus*, *Candidatus Liberibacter asiaticus* and *Candidatus Liberibacter americanus* - of the citrus HLB, also known as greening citrus disease (Bové, 2006; Gottwald, 2010). HLB is a destructive disease of citrus caused by these fluem-restricting bacteria, affecting yield in infected citrus trees and even leading to their death, which has already resulted in great citrus production yield losses worldwide (Bové, 2006; Yang et al., 2016). Transmitted from tree to tree by two citrus psyllid insect vectors – *T. erytreae* and *D. citri* – HLB is one of the most complex diseases of citrus as many factors pose an obstacle to its effective control. This disease is the result of interactions between pathogen, vector, hosts and the environment in its broadest definition (weather, soils, plant nutrition, presence of other pathogens and pests, etc.). Additionally, decision making from regulatory agencies and researchers concerning this disease is further difficulted by the inability to culture the causal organism, the lack of any known sources of natural resistance and its long latent period (da Graça et al., 2016).

Although some symptoms such as yellow shoots, lopsided fruits, color inversion, blotchy mottle, uneven fruit coloration and size and aborted seeds are characteristic of HLB, they do not always occur together on the same tree and can be distorted or masked by symptoms of other citrus diseases or some nutritional deficiencies, making the identification of this disease more difficult (Figure 1) (Bové, 2006; da Graça et al., 2016).

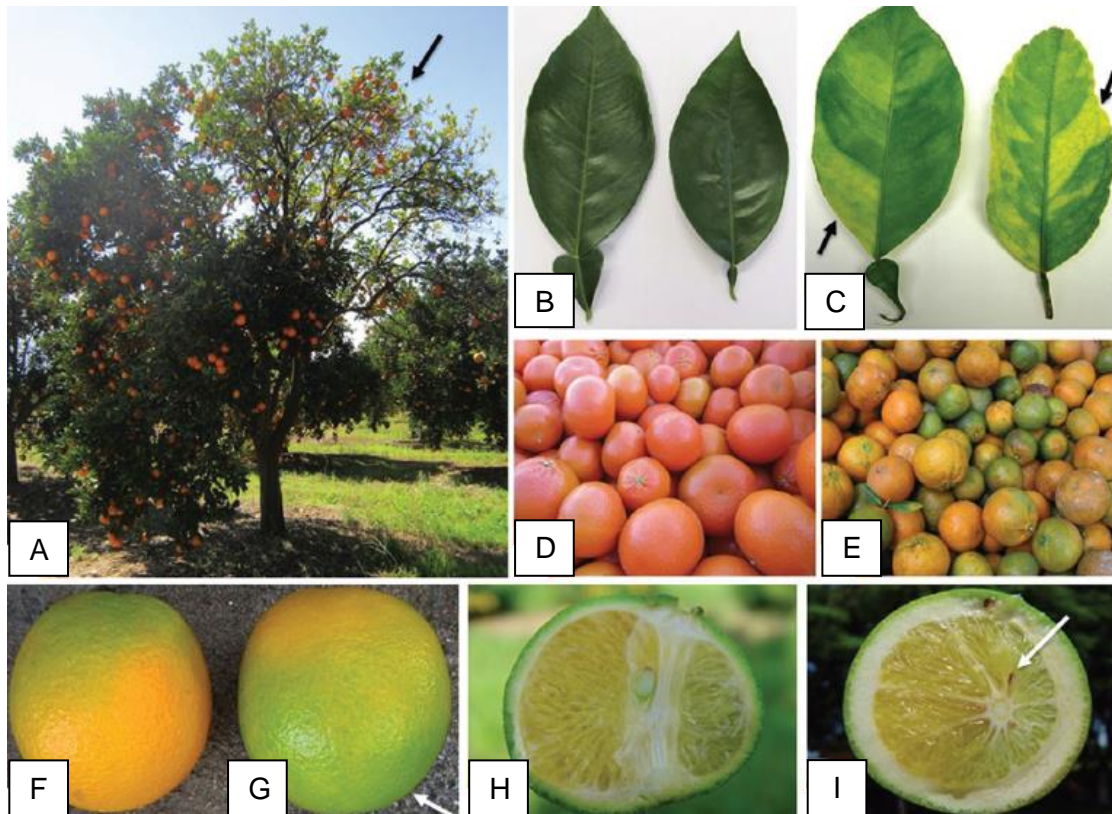


Figure 1 – Huanglongbing (HLB) Symptomatology. The HLB disease can be manifested in trees canopies, leaves, and fruits. (B), (D) and (F) illustrate healthy leaves and fruits for comparison. HLB can be detected by observing its symptomatology: (A) Yellow shoot; (C) Blotchy mottle; (E) Uneven fruit size and coloration; (G) Color inversion; (H) Lopsided fruit and (I) Seed abortion. Adapted from da Graça et al. (2016).

Apart from *T. erytreae*, only *D. citri* is other known biological vector of the citrus greening disease (Alves et al., 2014). Both have greatly affected citrus production in the Americas, Asia, and some parts of Africa (Antwi-Agyakwa et al., 2019).

2.2.2. Origin and current distribution

Cocuzza et al. (2017) suggests that *T. erytreae* is probably native from southeastern Africa and is nowadays present throughout the Afrotropic ecozone, invading Madeira archipelago in 1994 and the Canary Islands in 2002. The species has remained confined to these non-continental areas of Europe until August 2014, when it was observed in the province of Pontevedra, northwestern Spain. In the subsequent months, *T. erytreae* was spotted in the autonomous community of Galicia and in the district of Porto, Portugal (Figure 2) (Cocuzza et al., 2017). In the following years, a growing number of newly affected areas were registered throughout the Iberian Peninsula, with progressively more severe attacks. The lack of effective control and management measures previews the spread of the pest across Europe (Arenas-Arenas et al., 2018). Despite preventive efforts

to contain the dispersal, *T. erytreae* distributed widely in the coastal region of Portugal, recently reaching Alentejo and Algarve (DGAV, 2021). In Portugal, the Porto region, along with Montalegre, Vila Real, Aveiro and Coimbra, presents the most favorable environment to the species survival, as the climate offers fewer lethal days, and lower vapor pressure deficits (Paiva et al., 2020). Although *T. erytreae* tends to prefer a cooler climate with higher atmospheric moisture (Green & Catling, 1971), the transversal distribution of the pest throughout very different regions suggests a great capacity in adapting to a variety of environmental conditions such as equatorial, arid, and warm temperate climates with different temperatures and rainfall (Cocuzza et al., 2017).

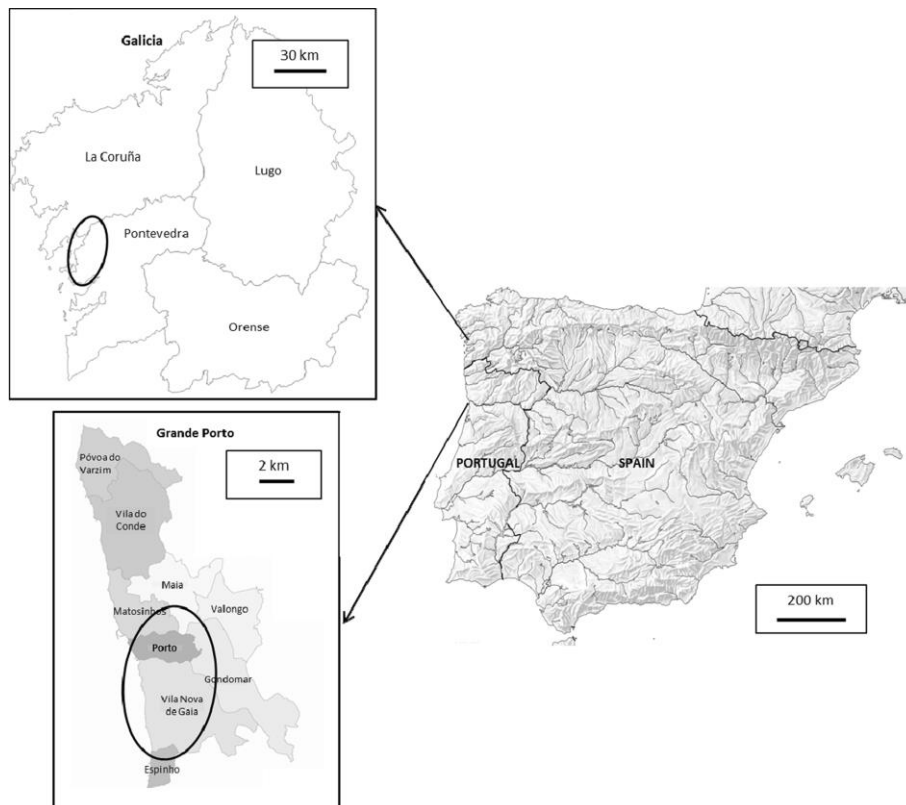


Figure 2 – Distribution of *T. erytreae* in the North of the Iberian Peninsula. Illustration from Cocuzza et al. (2017).

While no HLB has been identified in Europe and the Mediterranean Basin until this day, all the other main citrus-growing regions of the world except for Australia have already been infested by the pathogen. Just as the spread of its vector, the spread of the pathogen is also not contained, as evidenced by its recent expansion to previously HLB-free citrus areas in the Arabian Peninsula and in the equatorial and Western Africa (Dionisio et al., 2021). The Mediterranean Basin is the second major citrus-growing area worldwide and the largest citrus commercial importer and exporter center, therefore, containment of *T. erytreae* spread in this region is of great concern as it can easily pave

way for future HLB range expansions (Dionisio et al., 2021; Urbaneja-Bernat et al., 2020).

2.2.3. Biology and life cycle

T. erytreae life cycle is heavily influenced by both the climate conditions, typically preferring a humid and cooler climate (Green & Catling, 1971) and the availability of young shoots which often determines the number of laid eggs as well as the success of nymphs development and survival (Moran & Buchan, 1975). Wind may also play a role in the distribution densities of *T. erytreae* in the orchards as a higher number of psyllids was observed in the edges of orchards, especially those that are close to a windbreak (Van den Berg et al., 1991).

Regarding the host plants of this pest, plants of the Rutaceae family are generally recognized as the preferred hosts. Indigenous Rutaceae *Vepris lanceolata* and *Clausena anisata* are thought to be the original host plants, as the life cycle of the psyllid was significantly faster with a low mortality when reared on these plants (Moran, 1968). Nonetheless, *T. erytreae* can also commonly be found in *C. limon*, *Citrus jambhiri*, *Citrus medica*, *Citrus aurantiifolia*, *Citrus sinensis*, *Citrus reticulata*, *Citrus paradisi*, *Citrus maxima* and *Citrus unshiu* (Cocuzza et al., 2017). This clear preference for lemon plants was also observed by Benhadi-Marín et al. (2021). Morphometric characteristics of *T. erytreae* adults are also influenced by the host plant, which may play a role in the patterns of this insects geographical dispersal (Aidoo et al., 2019)

This small psyllid's life cycle has three main stages: egg, nymph and adult. Egg and nymphal development can take up to 38 days in summer and 47 days in winter until adult development, whose lifespan records for 73-82 days (Van den Berg, 1990).

Soon after mating, females actively seek adequate sites for oviposition of eggs on young leaves or at the tip of shoots, always preferring softer leaves (Annecke, 1963; Moran & Buchan, 1975). *T. erytreae* is a highly fecund species, and can lay more than 800 eggs per female after a single mating and more than 1300 eggs after continuous mating (Catling, 1973). Eggs can also be laid on mature leaves and flower petals albeit rarely and mostly during strong infestations, resulting in lower rates of survival (Moran & Blowers, 1967; Van den Berg, 1990). Embryonic development time is heavily dependent on temperature conditions. Typically, in ideal temperatures around 21-27 °C, eggs can take 6 to 13 days to fully complete their development (Annecke, 1963; Catling, 1973; Moran & Blowers, 1967; Van Den Berg & Deacon, 1992). *T. erytreae* eggs are yellow to

dark orange, pear-shaped and smooth with a sharp anterior point and a short posterior stalk, corresponding to the point of attachment into plant tissues with a water-absorbing function (Figure 3) (Moran & Blowers, 1967).

The eggs laid on shoots soon develop into nymphs following the leaves growth. On hatching, the first instar nymph looks for the appropriate feeding place, typically in soft stems and shoots and on the underside of young leaves (Moran & Blowers, 1967). During its feeding and development, a small gall begins to protrude on the upper surface of the leaf, increasing in size with nymphal growth (Van den Berg & Thomas, 1991). The development of juvenile stages is also dependent on temperature, humidity, and vegetative conditions. Nymphal cycle can only be completed in temperatures above 10 °C (Catling, 1973) and below 32 °C with high relative humidity (Green & Catling, 1971; Moran & Blowers, 1967). Optimal nymphal developmental temperatures seems to be in the 20-25 °C range, as noted by Catling (1973), which registered the time of development at an average temperature of 25 °C to be 17 days. Additionally, Van Den Berg and Deacon (1992) reported that at a temperature of 20.7 °C, nymphs take less than 20 days to complete their development, and Moran and Blowers (1967) recorded a period of 27 and 23 days for the development at 21 and 27 °C, respectively. Nymphs in the last fifth instar take the longest to complete their development, taking about 6 days to start molting to adults (Van Den Berg & Deacon, 1992).

Nymphal stage comprises five development instars (N1-N5) which are mostly sedentary but can travel for small distances if disturbed. These stages are very similar but can be differentiated by observing the number and length of the small waxy filaments around their body as well as other morphological differences. The N1 instar has a distinctive head shape, a small number of waxy filaments and a lack of wing buds. The N2 and N3 instars are more difficult to distinguish from each other apart from their discrepancy in size and number of filaments, while the N4 and N5 instars are easily recognizable by the appearance of two pale brown spots on the nymph's dorso upon molting to the N4 stage (Moran & Blowers, 1967) (Figure 3).

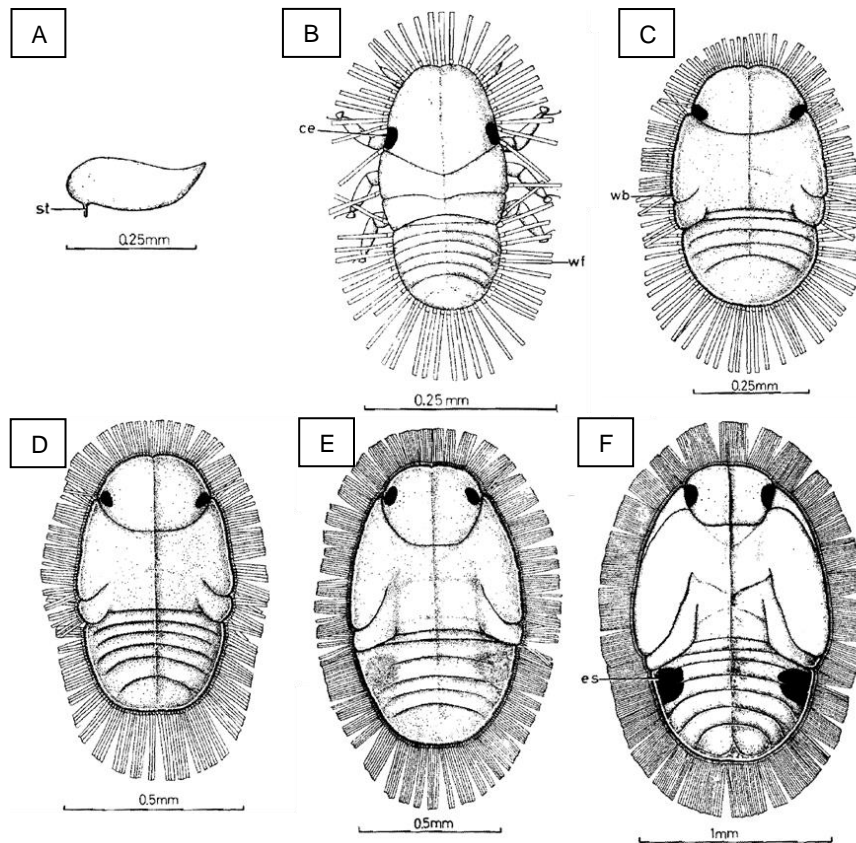


Figure 3 - Immature stages of *T. erytreae* life cycle with their respective width. Sizes not to scale. (A) egg in lateral view, with stalk “st”; (B) N1 instar nymph evidencing the presence of the compound eye “ce” and waxy filaments “wf”; (C) N2 instar nymph, with wing buds “wb” starting to form; (D) N3 instar nymph; (E) N4 instar nymph; (F) N5 instar nymph with its characteristic brown spots “es”. Illustration adapted from Moran and Blowers (1967).

Adults of *T. erytreae* are typically 2 mm in length, although females can be slightly larger than males (Van den Berg, 1990). Morphometric characteristics, particularly wing shape and size of *T. erytreae* adults is also related to the host plants in which its immature stages develop. Adults reared on *C. limon* and *Citroncirus spp.* plants are typically larger (Aidoo et al., 2019). Adults color ranges from green upon emergence and dark brown after completing sclerotization, usually after 5 days. After 10 days malpighian tubes can be observed through the dorsal abdomen (Van den Berg, 1990). Males and females can be easily distinguished by observing the shape of the abdomen and genitalia of adult forms: male genitalia possesses two lobiform basal projections directed backwards in the proctiger, while the female proctiger has a sharp concavity towards the base in the dorsal profile (Figure 4) (Cocuzza et al., 2017). Adults start mating as soon as their teneral hardens, however, the development of the first mature eggs can take from 3 to 14 days (Catling, 1973; Van der Merwe, 1923). Females typically mate two – four times a day (Catling, 1973).

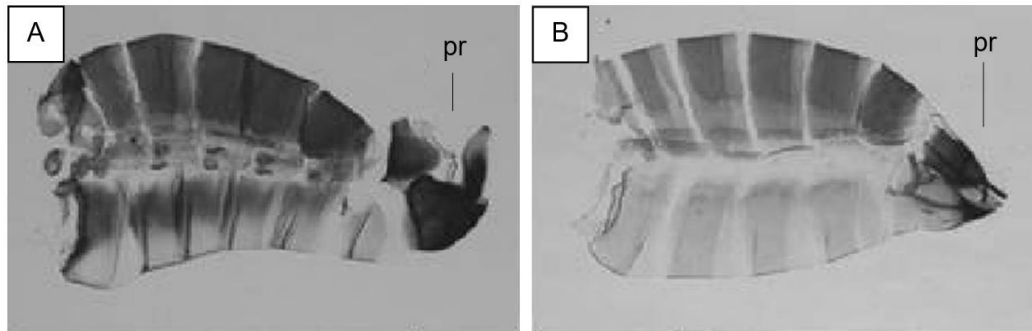


Figure 4 – Male (A) and female (B) abdomen of *T. erytreae* adults in lateral view, evidencing the differences in the proctiger “pr”. Adapted from Cocuzza et al. (2017).

Both nymphs and adults feed on plant sap but it is nymph’s feeding behavior and development which causes leaves distortion, a clear sign of this pest’s attack (Van den Berg, 1990). In typical citrus production sites, direct damage caused by this psyllid does not produce yield losses. Nonetheless, in plant nurseries, where plants are smaller and contain a higher number of shoots, high population levels of *T. erytreae* may lead to a reduction in the plants’ growth and even death (Tamesse & Messi, 2002). Indirect damage is more prevalent and may be observed in plants infested with this pest. Adults and nymphs feeding produces honeydew, a sugary substance that often falls on leaves and fruits, stimulating the growth of fungi. Honeydew also attracts ants to the citrus trees, which may protect the pest from its natural enemies, disrupting biological control (Cocuzza et al., 2017).

The knowledge of *T. erytreae* life cycle, its preferred hosts and its adequate development conditions serves as the basis for the research of new alternatives for its control.

2.2.4. Control methods

Despite the symptoms associated with its presence, *T. erytreae* was considered for a long time a secondary pest since no alteration or loss of physiological functions of the leaf is known as the result of an attack (Van der Merwe, 1923). Regardless, citrus greening disease has no known cure so, currently, the management is aimed at the vector (Belasque Jr et al., 2010).

T. erytreae populations can’t expand quickly, only moving from tree to tree when looking for new feeding and oviposition sites (Samways & Manicom, 1983), as such, rigorous application of quarantine measures is the first line of defense to control this pest dispersal (Cocuzza et al., 2017). Human activity is thus the main reason why *T. erytreae* can be found in regions far from those where it is endemic, particularly due to the careless

movement of material from affected to unaffected areas (Cocuzza et al., 2017). Quarantine measures have been proactively established by the Portuguese Government since the species' detection in the territory, with regular updates on the affected areas and the implementation of buffer zones (DGAV, 2021) (Figure 5).

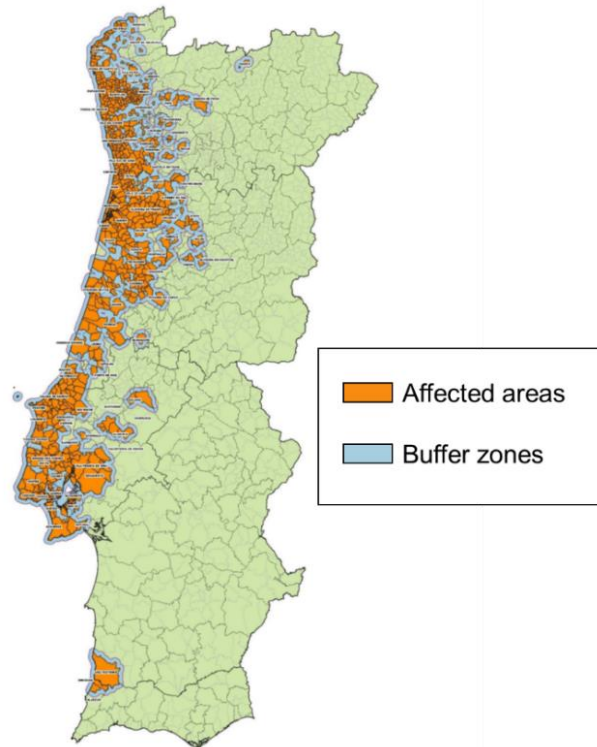


Figure 5 - Defined zones for the control of *T. erytreae* in Portugal, presenting the affected areas, where presence of the psyllid has been registered and buffer zones, corresponding to the surrounding zones of these areas in a 3 km range. Adapted from DGAV (2021).

The implementation of effective control practices with insecticides proves difficult due to the struggle in applying these substances in private areas such as private gardens or family orchards (Gottwald, 2010). These places are common throughout the northern Iberian Peninsula and often act as a refuge for the insect and sources of resurgent outbreaks (Cocuzza et al., 2017). As such, usage of insecticides may be an useful approach to control *T. erytreae*, but only as a measure to keep its populations at a low level.

In Africa, places with numerous *T. erytreae* populations often use broad-spectrum pesticides, such as systemic chloronicotinyl insecticides in an attempt to control the pest. In Europe the most used and effective insecticide was the neonicotinoid thiamethoxam, but it is banned for usage since May 2018. Nonetheless, remaining neonicotinoid compounds acetamiprid and imidacloprid are still effective insecticides on *T. erytreae*, even though the latter is not allowed in open-field conditions (Dionisio et al., 2021).

Cultural control is common practice and makes use of *T. erytreae* temperature and humidity vulnerability to control its spread. Cultivars with reduced and well-defined flushing periods can be grown in areas with high temperatures and low humidity, adversely affecting the psyllid dispersal (Van den Berg, 1994). Practices/fertilization that can facilitate plant stress, and thus stimulate vegetative growth, should be avoided and removal of abandoned or unproductive citrus trees is also encouraged (Cocuzza et al., 2017). Removal of indigenous host-plants present near citrus orchards is also advised since they may serve as natural refugees for the psyllid (Van den Berg et al., 1991). Application of specific size anti-insect meshes also seems to be effective in blocking *T. erytreae* and its usage should be encouraged especially to avoid its entrance in citrus-growing nurseries, intensifying the efforts to prevent the psyllid's spread from infested to non-infested areas (Urbaneja-Bernat et al., 2020). Traps with yellow-green fluorescent bands in the 530 nm spectrum may also be utilized as a sensitive indicator of psylla abundance (Samways, 1987). The spray application of kaolin has also shown to be effective for *D. citri* control, where orchards sprayed with kaolin (2%) at 7-14 days intervals had a significant reduction of *D. citri* infestations (Miranda et al., 2021). Due to the similarities between both insects, the applicability of the same solution on *T. erytreae* could also be possible.

Biological control of *T. erytreae* is a valuable option. There are many *T. erytreae* natural enemies, including several generalist predators, predatory mites and occasional predators like syrphids (Catling, 1970) and coccinellids (Samways, 1984). Other insects belonging to Hemiptera (Reduviidae, Pentatomidae, Anthocoridae, Lygaeidae and Miridae), Neuroptera (Chrysopidae and Hemerobiidae), Diptera (Syrphidae), Hymenoptera (Formicidae), Dermaptera (Forficulidae), Arachnida and Acarina as well as some spiders and lacewings, were also recorded as predators of *T. erytreae* (Hernández, 2003; Molina et al., 2021; Van den Berg & Anderson, 1987). Although these predators can contribute to reducing the size of *T. erytreae* populations and are naturally present in the Mediterranean basin (Cocuzza et al., 2017), they have not been found to significantly contain its growth (Catling & Annecke, 1968; Van den Berg et al., 1992). On the other hand, *T. dryi* (Hymenoptera: Eulophidae) is a nymphal parasitoid native from South Africa and is regarded as the most effective parasitoid of *T. erytreae* (Mc Daniel & Moran, 1972; Van den Berg & Greenland, 2000), successfully controlling its populations on islands like Reunion Islands and Mauritius in only a few years (Aubert, 1987; Etienne & Aubert, 1980). Other parasitoids are identified as potential candidates for usage as biological control agents, these include *Psyllaephagus pulvinatus* and a newly identified parasitoid from *Tamarixia* genus (Pérez-Rodríguez et al., 2019). Although these

parasitoids appears to be a valuable option, their usage needs to consider that in some regions their impacts on *T. erytreae* populations may be hindered by hyperparasitoids (Annecke & Moran, 1982; Mc Daniel & Moran, 1972). *Syrphophagus cassatus* seems to be the most relevant hyperparasitoid and caution must be taken to prevent the accidental introduction of this parasitoid when importing *T. dryi* to Europe (Cocuzza et al., 2017; Pérez-Rodríguez et al., 2019).

Recently, susceptibility of *T. erytreae* to some strains of entomopathogenic fungus has also been observed by Aidoo et al. (2021).

In conclusion, to control *T. erytreae*, various methods are applied, such as farm sanitation with synthetic chemicals, and controlled release of the nymphal parasitoid, *T. dryi*, as a biological control method (Van den Berg et al., 1991). These approaches have not yet been proven effective in the long term (Antwi-Agyakwa et al., 2019), and although the usage of insecticides together with preventive cultural measures can show good results, they may not be effective in controlling *T. erytreae* and HLB, especially in small-scale farms (Belasque Jr et al., 2010; Kilalo et al., 2009). Furthermore, as mentioned, insecticide usage can cause increased vector resistance, harmful effects on non-target species, environmental contamination and reduction of non-target beneficial organisms and insects, including beneficial predators and parasitoids (Antwi-Agyakwa et al., 2019; Desneux et al., 2007; Naqqash et al., 2016; Stoytcheva, 2011). The usage of the nymphal parasitoid *T. dryi*, although a valuable option, has only proved its viability in rather isolated regions, surrounded by water, and its continental effectiveness can only be correctly determined when parasitoid-pest populations are balanced. Furthermore, its usage is also further threatened by the eventual introduction of hyperparasitoids (Pérez-Rodríguez et al., 2019). Additionally, even though *T. erytreae* populations can be reduced by using these containment measures, its effectivity on avoiding the arrival of HLB is still up for debate. Therefore, to sustainably slow its expansion, it is urgent to look for new environmentally friendly alternatives to manage *T. erytreae*.

Such need for alternatives highlights the development of new options for the control of *T. erytreae*. EOs have shown to be viable options: their effects are well demonstrated against related species, and recent literature highlighted their potential application on *T. erytreae*. As such, it is the utmost utility to expand the knowledge on these compounds' effects, not only on the pest but also on its preferred host *C. limon*.

The acquired knowledge on the effects of EOs on other pests served as the basis to study the toxicity, repellency and ovicidal effects of select EOs active compounds on *T. erytreae*. The development of this works methodology also focused on assessing the

existence of phytotoxic effects of EOs on *C. limon*, a possible effect of the direct application of these compounds, as noted on other crops. Likewise, the applicability of these compounds in field conditions was considered when designing the experimental roadmap.

3. Materials and Methods

The procedures used in this work followed a sequential, evaluative, and selective order. Selection of EOs active compounds and preliminary assays were made preventively as to evaluate the compounds potential more effectively and efficiently, defining the concentrations to be tested while also permitting the adequate adjustments on the methodology to be applied. The biocidal potential of the selected compounds in the toxicity and phytotoxicity assays, was evaluated through the application of these compounds at several concentrations, permitting an evaluation of their dose-response. The active compounds concentrations to test in the repellent and ovicidal effect assays as well as the semi-field assay, were selected based on previous results from the toxicity bioassay and phytotoxicity assay, i. e. only concentrations that were used in the toxicity bioassay and did not pose phytotoxicity to plants were further applied to observe repellency (i.e. settling behaviour and posture deterrence), ovicidal effects and evaluate the insecticidal potential in semi-field conditions (Figure 6).

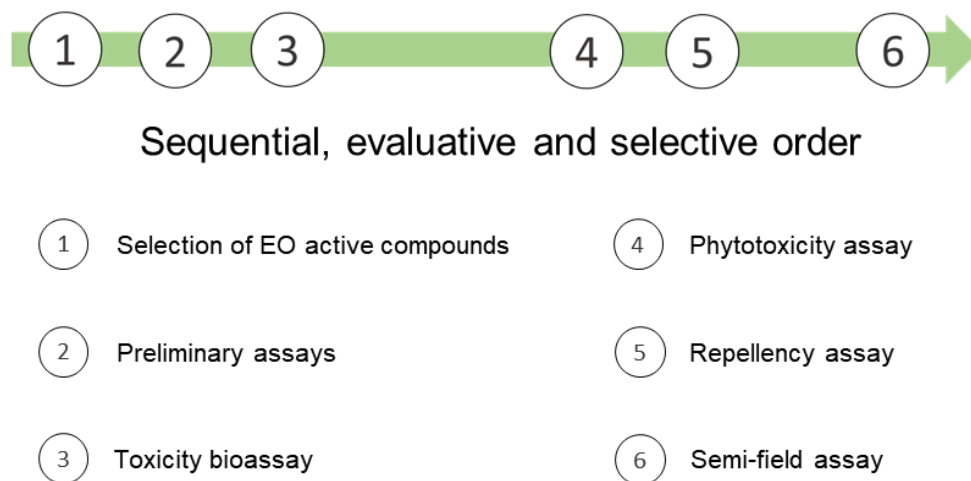


Figure 6 - Experimental design of this work, showing the different assays to be performed, in chronological order.

The next sections will describe in detail all materials and procedures utilized for the insect breeding and plant formation, EOs selection, preliminary assays and all the following tests employed to assess the insecticidal potential of the selected compounds.

All insect breeding and plant formation, as well as all assays related to *T. erytreae* manipulation were performed in Campus Agrário de Vairão, Faculdade de Ciências da Universidade do Porto, located in Vairão, Vila do Conde district of Porto. Porto region qualifies as one of the quarantine zones in Portugal (DGAV, 2021), making it possible to

responsibly breed *T. erytreae* in all its biological cycle forms, as well as perform all the described assays.

3.1. Insect rearing and plant provision

Insect rearing is an essential practice for the supply of insects to scientific entomological assays. These insects were produced in a controlled environment to know their age, the number of generations and warrant the best possible conditions for their survival and breeding.

In order to guarantee the correct execution of the assays planned for this study, a *T. erytreae* rearing has been maintained in a climatized insectary room and a semi-open greenhouse located in Campus Agrário de Vairão, Faculdade de Ciências da Universidade do Porto. In the insectary room, insects were maintained in entomological breeding cages, with potted *C. limon* plants prepared for this purpose. In the semi-open greenhouse, insects were maintained freely inside the greenhouse as well as inside entomological cages, all together with *C. limon* plants (Figure 7).



Figure 7 - Facilities used for *T. erytreae* breeding. (A) Cage present inside the insectary room, where psyllids are kept freely inside together with potted *C. limon* plants; (B) Semi-open greenhouse prepared as a rearing facility, psyllids were kept freely inside with *C. limon* at various vegetative stages, as well as in entomological cages allocated on the right.

In the breeding room, insects are maintained in *C. limon* plants (Eureka cultivar grafted in *C. aurantium*) with appropriate conditions of light (approx. 15000 lux), humidity (70±10%) and temperature (25±5 °C). The light is provided by several lamps arranged on the upper structure of breeding cages, and 4500 K and 6000 K LED lamps with an intensity of 1200 lumens are used, combined with LED Sunlike lamps, switched on in a

cycle of 14:10 h (L:D). These light conditions proved useful for maintaining the breeding capability but were the limiting factor, especially during winter months, with less natural sunlight entering the room.

Since *T. erytreae* is a sucking-biting insect, which depends on young buds for laying and developing immature stages, rearing requires a high synchronization between the vegetative stage of *C. limon* plants, temperature and humidity conditions, and the insect's life cycle stage. Plants kept in this room regularly show new buds and strong growth. The available plants (1-2 years old) kept in the cage were frequently pruned as needed and fertilized to stimulate their growth. Monthly fertilization (which proved to be essential for maintaining the plants' quality) was carried out with slow-release complex granulated blue fertilizer NPK (S) 12-8-16 (20) and through irrigation with a *Welgro Micromix*[®] solution.

All plants were watered regularly, 3 times a week and regularly pruned and fertilized, in cycles of 3 weeks to regularly stimulate new bud breaks.

In order to overcome the difficulty of imitating sunlight in the breeding room, as well as potentiate the number of insects bred, in February 2020 a greenhouse was donated to carry out an extension for *T. erytreae* rearing. To jump-start the new rearing facility, orange and lemon plants infested with *T. erytreae* adults from the field orchard of Campus de Vairão were set. Soon afterwards, *T. erytreae* adults were brought from the breeding room and new *C. limon* plants were placed inside replacing the previous ones. Plants from the greenhouse followed the same pruning and fertilizing treatment as those in the insectary room. The watering was done by sprinklers and programmed to water twice a day for about 15 minutes per watering in the summer, but was also adjusted according to the weather conditions foreseen for each week. This system also has the function of cooling and maintaining high relative humidity inside. The greenhouse, due to its small dimensions, reaches high temperatures during the summer so, in addition to the fog system provided by the sprinklers a shading coverage was placed on the roof of the greenhouse to provide a cooler environment during these months. Temperatures inside the greenhouse ranged from 18 °C to 28 °C but reached 36 °C at their highest during the summer.

Due to the high number of plants required for the phytotoxicity, repellency and ovicidal effect assays (approximately 130 plants), a plant provision plan was started in May 2020. To assure equal characteristics between plants and schedule their age as needed for the assays, *C. limon* seeds were used for plant propagation. All the seeds were gathered from the lemon orchards on Campus Agrário de Vairão.

Seeds were collected from fruits and let dry for 3 days, after which the seed coat was removed, exposing the embryo. For germination, seeds were placed inside a dark box covered with wet cotton until radicle protrusion. Upon radicle protrusion, the seeds were collected from the recipient and placed inside seed trays and covered in turf-rich soil and watered daily. Seedlings were then kept inside the seed trays until reaching 10 cm of height and were then transferred into larger 0.5 L pots and further covered with turf enriched soil. At this point, these small plants started being fertilized similarly to the insect breeding plants, as described previously, and watered 3 times a week. During all the seed propagation process plants were kept at the *Aralab*[®] Walk-in 13000e phytoclimatic chamber at a temperature of 25±2 °C, relative humidity of 70±5% and 14:10 h (L:D).

3.2. Selection of EOs active compounds and preliminary assays

The choice of the EOs active compounds was determined by its toxicity in *T. erytreae* taxonomically related insects, as well as its availability and potential for commercial usage in agricultural production sites (Section 2.1.6. and 2.1.7.). As such, Eugenol and Pulegone were the selected EOs active compounds for testing.

Eugenol, 99% was purchased from *ACROS Organics*[®], Geel, Belgium whereas (R)-(+)-Pulegone, 85% technical grade was purchased from *Aldrich*[®], Steinheim, Germany.

Preliminary assays were made before the assessment of the direct contact toxicity of treatments. The implemented direct contact toxicity bioassay utilizes solvents in order to properly dissolve the oil and reach the desired concentrations to be tested. These solvents must dissolve the compounds while not causing great mortality by themselves on the testing subjects. Due to the foreseen need for a high number of psyllids for the selected assays, differences in susceptibility between both insectary populations needed also to be assessed. Also, the evaluation of lethal dosages requires many times the usage of several concentrations before even starting to notice effects on the mortality of the testing subjects, as such, testing concentrations needed to be determined.

Given these prepositions, the goal of these preliminary assays was to preventively:

- i) determine appropriate dilution solvents;
- ii) verify possible differences in susceptibility between insectary room kept and greenhouse psyllids;
- iii) Narrow down testing concentrations.

These assays followed the topical application procedure, as such, a detailed description will be allocated in the next section. This procedure will be transversal to all the preliminary assays as well as the toxicity bioassay and should therefore be taken as the reference procedure in these assays.

3.2.1. Topical application procedure

The topical application procedure tested the mortality of each selected compound through direct contact topical application on N4-N5 instar nymphs dorsal-thorax region.

Each assay starts by collecting leaves infested with N4-N5 instar nymphs from either the room and greenhouse insectary, depending on the assay. From each leaf, 22 nymphs are then cautiously transferred into the bottom lamina of a young and tender healthy *C. limon* leaf with the aid of a straight fine dissection needle. Leaves petioles are then inserted in a 2 mL 1.50% agar-filled microtube to keep turgescence, where they are kept until each solution was topically applied.

The application of each testing solution is made through the observation of the test subjects on a binocular lens. Leaves are then placed in a glass petri dish with the bottom lamina facing upwards. With the aid of a 10 μ L microsyringe (*Innovative Labor Systems*[®] GmbH, Stützerbach, Germany) 0.5 μ L of each testing solution are then carefully applied to the dorsal-thorax region of each nymph (Figure 8).

After topical application, each leaf's petiole is gently sliced at a length of at least 1 cm in a bevel shape. Using dissection tweezers, leaves are then picked by the petiole and transferred into a translucent recipient with 1.50% solidified agar solution and inserted in the agar by the petiole, keeping the leaf in a vertical position. Due to the volatile nature of the essential oils and to allow the leaves to breathe, containers are covered with tulle fabric secured with elastic bands (Figure 9).

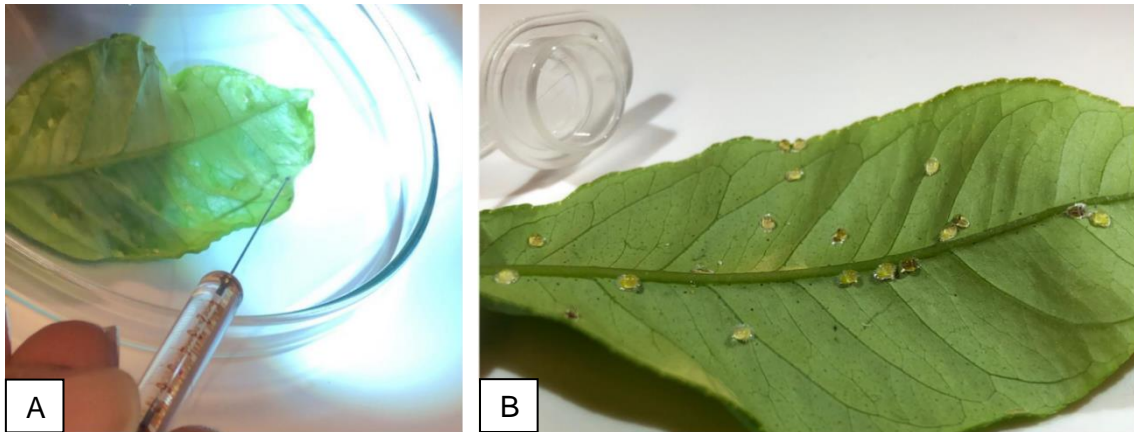


Figure 8 - Topical application procedure on *T. erytreae* nymphs. (A) Through the binocular lens and with the aid of a micro syringe, 0.5 μ L of the testing solution is applied to the dorsal-thorax region of each nymph, with only the drop contacting their body. (B) After application, the drops of each tested solution can be observed resting in the nymph's dorsal region.

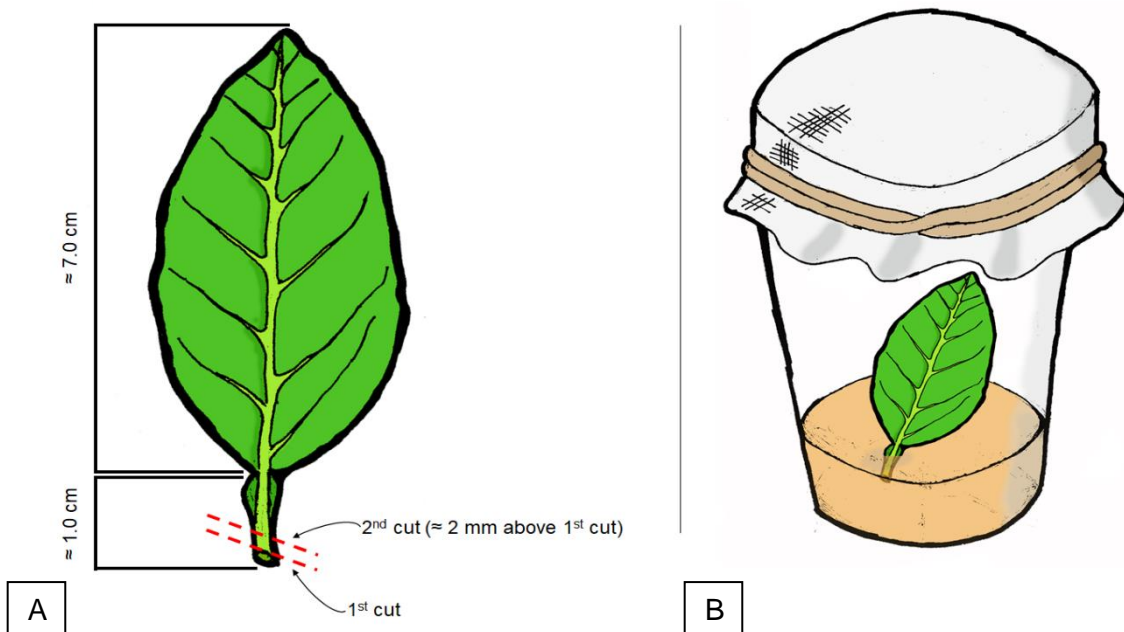


Figure 9 – Representation of the methodology applied to maintain nymphs in the leaves during the period of the assay. (A) schematic representation of the citrus leaves utilized in the assay, indicating their size as well as the petiol's. 1st and 2nd cuts are made in the petiole at a bezel shape before inserting the leaf in agar solution. (B) Leaf resting vertically inside the container, with petiole submerged in 1.50% agar solution. The container is covered in tulle fabric and secured by elastic bands.

Each infested leaf served as a replicate and were kept at 25 ± 2 °C, $70 \pm 5\%$ RH and 14:10 h (L:D) in the phytoclimatic chamber (*Aralab*[®] Walk-in 13000e). Mortality was registered twice, after 24 h and 48 h, with the aid of a binocular lens. Nymphs were considered dead when no movement was observed for 30 seconds when probed and rotated with a straight fine dissection needle.

When accounting for mortality, leaves had to be removed from the recipient to be observed in the binocular lens. As such, to keep turgescence while observing, petioles were always inserted in a 1.50% agar-filled microcentrifuge tube. After the first observation, the petiole was again cut at the same angle, 2 mm above the first cut. The leaf was then returned to the recipient, which is once again covered and maintained at the same conditions until the second observation, when mortality was equally registered following the same methods applied in the first observation (Figure 9).

3.2.2. Selection of dilution solvents

To properly achieve the desired testing concentrations, the selected compounds for the study need to be correctly dissolved. The EO active compounds selected for these assays are immiscible in water, as such a solvent or an emulsifier is needed. From the literature review, various options can be used as EOs solvents and emulsifiers, such as *Tween*[®] 80 (Papadimitriou et al., 2019), *Tween*[®] 20 (Mota et al., 2017), acetone (da Silva et al., 2020; Gaire, Scharf, et al., 2020; Huang et al., 2002; Tian et al., 2015) and dimethyl sulfoxide (Almadiy, 2020). Since *Tween*[®] 80 and Acetone (>99%) were readily available (VWR[®], Fontenay-sous-Bois, France), these were selected for solution preparation.

To be eligible for usage in this work, the selected solvent needed to make sure to meet two criteria in the following order:

- i) The solvent couldn't by itself exert significant toxicity on the testing subjects;
- ii) After dissolving the oil at the highest testing concentration, a homogeneous and clear solution was required to be obtained, with no visible phase separation.

Firstly, to evaluate the possible usage of the two selected solvents and observe their toxicity, various solutions were prepared:

- Solvent 1 (S1): *Tween*[®] 80 0.01% in distilled water:acetone (80:20 v/v);
- Solvent 2 (S2): *Tween*[®] 80 0.01% in distilled water (v/v);
- Solvent 3 (S3): Acetone 20% in distilled water (v/v);
- Solvent 4 (S4): Acetone 60% in distilled water (v/v);
- Solvent 5 (S5): Acetone 80% in distilled water (v/v);
- Solvent 6 (S6): Acetone 100%.

As such, 10 mL of each solution (v/v) was prepared by measuring each solvent with a graduated cylinder for volumes above 2 ml, and with a 2-20 µL micropipette (VWR[®]

Standard Line Pipettor, Leuven, Belgium) for smaller volumes. Solvents were then mixed in a test tube with the aid of a vortex mixer at 1800 rpm.

After each solution was prepared, 0.5 µL was topically applied to 18-22 N4-N5 instar nymphs per replicate (4 replicates) with the aid of a 10 µL microsyringe following the same topical application procedure described in Section 3.2.1.. Mortality was observed after 24 h. Mortality rate (MR), mean and standard deviation were calculated using *Microsoft® Office Excel*.

The observed mortality for S4 was less than 20% for both 24 h and 48 h after application. The observed mortality was then in line with the requirements desirable for mortality tests in insecticides (WHO, 2018). The same solution also correctly and thoroughly dissolved Eugenol and Pulegone at 6.40% concentrations, as such, 60% Acetone was selected as the dilution solvent and thus the Negative Control for posterior assays.

3.2.3. Verifying differences in susceptibility between populations

Due to the large number of psyllids required for the assays and to assure a constant flow of biological material, the feasibility of using both insectary room and greenhouse nymphs needed to be assessed. Due to possible genetic differences that could impose different levels of resistance between the psyllid populations, an assessment of their susceptibility to the testing compounds was essential. To verify such possible differences, a toxicity bioassay using the same topical application procedure described in Section 3.2.1. was employed.

This experiment was conducted simultaneously to the selection of dilution solvents, as such, previously described (Section 3.2.2.) solvents S1, S2 and S3 were applied to 32-43 N4-N5 instar nymphs from each group - insectary room nymphs (IN) and greenhouse nymphs (GN) - and mortality was recorded after 24h. Mean and standard deviation were calculated using *Microsoft® Office Excel*.

These preliminary results show that IN and GN may both be used for the toxicity bioassay. However, for better interpretation of the final results, the usage of only one population should be prioritized. Therefore, GN were selected for usage in the toxicity bioassay since they can be slightly less susceptible to the action of the tested solvents.

3.2.4. Selection of testing concentrations: range-finding bioassay

In toxicity bioassays, LD₅₀ values may range widely. These dosages are dependent on the testing compound, methodology applied and the testing subject. In this work, LD₅₀ and LD₉₀ values of Eugenol and Pulegone on *T. erytreae* were to be obtained through topical application of these compounds in different concentrations on N4-N5 instar nymphs. On *T. erytreae* no available data on its susceptibility to EOs was available at the period of testing, as such, a prospection of the possible testing concentrations needed to be made.

To determine the starting test concentrations, firstly, a throughout research on direct contact mortality and LD₅₀ values of the testing compounds (Eugenol and Pulegone) on taxonomically related insects was made. Afterwards, by using the same topical contact procedure described (Section 3.2.1.), range finding bioassays were conducted to outline the testing concentrations that would be expected to carry mortality on the testing subject.

Using *Web of Science*[®] with the search criteria “All databases, Mentha pulegium or Pulegone AND oil* AND insecticide*” as well as “All databases, Syzygium aromaticum or Eugenol AND oil* AND insecticide*”, articles containing information on the direct contact toxicity of these essential oils across multiple insect species were selected. Collected LD₅₀ toxicity units were then transformed into v/v concentrations for easier comparison (Table 1 and 2). Afterwards, in a cross-comparative approach, concentrations for the range-finding tests were chosen based on two main criteria:

- i) Usage of direct contact toxicity assays, i. e. microapplication and/or spray;
- ii) Testing on taxonomically related insects i. e. Hemiptera order and preferably Psyllidae and Triozidae families.

Following the gathered data (Table 1 and 2), 0.05% for Pulegone and 0.40% for Eugenol were chosen as the starting concentrations for the preliminary range-finding assay.

Due to the limited number of available nymphs, it was decided to make a preliminary range-finding assay using the topical contact procedure, albeit with a smaller number of nymphs (N=40). This assay would start with the previously selected concentrations (0.05 % for Pulegone and 0.40 % for Eugenol) and increment the dosages by a 2x multiplier per test until mortality reached 20%. The goal of such preliminary testing is to determine the concentration at which the compound starts to exert a toxic effect on the testing subject, preventing the unnecessary usage of *T. erytreae* nymphs.

With the aid of a 10 µL microsyringe, 0.5 µL of the prepared solutions were topically applied to 20 N4-N5 instar *T. erytreae* nymphs following the procedures described in Section 3.2.1. and mortality was registered after 24 hours.

Testing solutions were prepared on a v/v basis. Volumes were measured with the aid of a 2-20 uL micropipette for volumes in the 0-2 mL range, and a 10±0.2 mL glass graduated cylinder for volumes over 2 mL. The EOs active compounds (Eugenol and Pulegone) were diluted with acetone in a glass test tube with the aid of a vortex mixer at 1800 rpm.

After the first tested concentration for Eugenol (0.40%), 21 % MR was observed. As for Pulegone, four different concentrations needed to be tested (0.05%; 0.10%; 0.20%; 0.40%) before mortality reached 20%. Following this preliminary testing, a starting concentration of 0.40% for both Eugenol and Pulegone was selected.

Table 1 - Resumed table of the references found in *Web of Science*[®] search for “Eugenol” and “*Syzygium aromaticum*” search criteria. Direct contact toxicity assays and Hemiptera order of species are highlighted in bold. MR stands for Mortality Rate.

Reference	Scientific name	Order	Family	Assay	LD ₅₀	LD ₅₀ % (v/v)
(Tian et al., 2015)	<i>Cacopsilla chinensis</i>	Hemiptera	Psyllidae	Microapplication	1.67 (µg.nymph ⁻¹) 0.67 (µg.adult ⁻¹)	0.31% 0.13%
(Almadiy, 2020)	<i>Aedes aegypti</i>	Diptera	Culicidae	Dip in solution	23.6 µL.L ⁻¹	0.002%
(Gaire, Lewis, et al., 2020)	<i>Cimex lectularius</i> L.	Hemiptera	Cimicidae	Microapplication	52 µg.mg-body-weight ⁻¹	19.6%
(da Silva et al., 2020)	<i>Musca domestica</i>	Diptera	Muscidae	Moistened paper disk Microapplication	2.5 mg.mL ⁻¹ - 75% MR 30 mg.mL ⁻¹ - 30% MR	0,24% - 75% MR 2.83% - 30% MR
(Huang et al., 2002)	<i>Sitophilus zeamais</i> <i>Tribolium castaneum</i>	Coleoptera Coleoptera	Curculionidae Tenebrionidae	Microapplication	31 µg.mg ⁻¹ 30.7 µg.mg ⁻¹	17.2% 12.6%
(Ghafoor et al., 2019)	<i>Drosicha mangiferae</i>	Hemiptera	Pseudococcidae	Dip in solution	6.31% (v/v)	6.31%
(Mota et al., 2017)	<i>Aleurodicus cocois</i>	Hemiptera	Aleyrodidae	Spray application	3.63 mg.mL ⁻¹	0.59%

Table 2 - Resumed table of the references found in *Web of Science*[®] search for “Pulegone” and “*Mentha pulegium*” search criteria. Direct contact toxicity assays and Hemiptera order of species are highlighted in bold. MR stands for Mortality Rate.

Reference	Scientific name	Order	Family	Assay	LD ₅₀	LD ₅₀ % (v/v)
(Pavela, 2008)	<i>Musca domestica</i>	Diptera	Muscidae	Microapplication	13 µg.fly ⁻¹	1.4%
(Pavela, 2005)	<i>Spodoptera littoralis</i>	Lepidopterae	Noctuidae	Microapplication	0.1 µL.larvae ⁻¹ : 29% MR	5%: 30% MR
(Franzios et al., 1997)	<i>Drosophila melanogaster</i>	Diptera	Drosophilidae	Moistened paper disk	0.17 µL.larvae ⁻¹	
(Pavlidou et al., 2004)	<i>Drosophila melanogaster</i>	Diptera	Drosophilidae	Moistened paper disk	0.17 µL.mL ⁻¹	0.017%
	<i>Bactrocera oleae</i>	Diptera	Tephritidae		0.09 µL.mL ⁻¹	0.009%
(Rim & Jee, 2006)	<i>Dermatophagoides pteronyssinus</i> and <i>D. pteronyssinus</i>	Acariformes	Pyroglyphidae	Moistened paper disk	0.03 µL.cm ² -1 - 98.7% MR; 0.013 µL.cm ⁻¹ - 2.6 % MR	0.063%: 98.7% MR; 0.031%: 2.6% MR
(Michaelakis et al., 2011)	<i>Culex pipiens</i>	Diptera	Culicidae	Dip in solution	27.23 mg.L ⁻¹	0.003%
(Papadimitriou et al., 2019)	<i>Tetranychus urticae</i>	Trombidi-formes	Tetranychidae	Spray application	500 µL.L ⁻¹ : 68.5% MR	0.05%
	<i>Aphis spiraecola</i>	Hemiptera	Aphididae		500 µL.L ⁻¹ : 40% MR	0.05%
	<i>Nesidiocoris tenuis</i>	Hemiptera			500 µL.L ⁻¹ : 20% MR	0.05%
(Behi et al., 2019)	<i>Aphis spiraecola</i>	Hemiptera	Aphididae	Moistened paper disk	0.478 µL.mL ⁻¹	0.048%
	<i>Aphis gossypii</i>	Hemiptera	Aphididae		0.547 µL.mL ⁻¹	0.055%

3.3. Evaluation of biopesticide potential of Eugenol and Pulegone

3.3.1. Toxicity bioassay

To assess the potential usage of Eugenol and Pulegone, and its mixture, as direct-contact insecticides, a topical contact bioassay was made applying the same methodology described in Section 3.2.1. For this, 15 treatments (five concentrations (0.40% (1), 0.80% (2), 1.60% (3), 3.20% (4) and 6.40% (5)) of Eugenol (E), Pulegone (P) and a 1:1 mixture of Eugenol + Pulegone (EP)) were prepared (Table 3). Acetone at 60% concentration was used as the Negative Control. *Confidor*[®], *Bayer*[®] *CropScience* (imidacloprid as the active substance) was used as the Positive Control at the commercially recommended 0.06% concentration.

These concentrations were prepared following a serial dilution method. First, for Eugenol and Pulegone solutions, two 5 mL 32 % concentration stock solutions were prepared in pure acetone on a v/v basis. For the Eugenol + Pulegone mixture, two other separate 5 mL solutions of Eugenol and Pulegone were prepared at a 16% concentration, also in

acetone (Table 3). The oils and acetone volumes were measured using a graduated 10 mL glass cylinder and mixed in a glass test tube with the aid of a vortex mixer at 1800 rpm. The prepared solution was then removed and transferred with a Pasteur pipette into hermetic glass flasks and stored in the freezer at 5 °C temperature.

For the following series of dilutions, quantities were also measured on a v/v basis. For each test concentration, 1 mL of solution was prepared. With the aid of a 20-200 µL micropipette, defined volumes of each stock solution and acetone were measured and transferred independently into a test tube. Distilled water was only added to each solution after acetone and stock solution were thoroughly mixed. Throughout the whole process, the solution was mixed frequently in a vortex mixer to assure its homogeneity. Prepared solutions were then removed using a Pasteur pipette and stored in 1 mL hermetic flasks at a 5 °C temperature.

Table 3 - Prepared concentrations for the toxicity bioassay, with the indication of the code used for each treatment. Volumes used for each compound in the solution are also described.

Solution	Final concentration (%)	Eugenol 32% Solution (µL)	Pulegone 32% Solution (µL)	Acetone (µL)	H ₂ O (µL)	Final volume (µL)
<i>Eugenol</i>						
E1	0.40	12.50	-	591.50	396.00	1000.00
E2	0.80	25.00	-	583.00	392.00	1000.00
E3	1.60	50.00	-	566.00	384.00	1000.00
E4	3.20	100.00	-	532.00	368.00	1000.00
E5	6.40	200.00	-	464.00	336.00	1000.00
<i>Pulegone</i>						
P1	0.40	-	12.50	591.50	396.00	1000.00
P2	0.80	-	25.00	583.00	392.00	1000.00
P3	1.60	-	50.00	566.00	384.00	1000.00
P4	3.20	-	100.00	532.00	368.00	1000.00
P5	6.40	-	200.00	464.00	336.00	1000.00
<i>Eugenol + Pulegone</i>						
		16%	16%			
EP1	0.40	12.50	12.50	579.00	396.00	1000.00
EP2	0.80	25.00	25.00	558.00	392.00	1000.00
EP3	1.60	50.00	50.00	516.00	384.00	1000.00
EP4	3.20	100.00	100.00	432.00	368.00	1000.00
EP5	6.40	200.00	200.00	264.00	336.00	1000.00
<i>Negative Control</i>						
		-	-	24.00	16.00	40.00
<i>Blank</i>						
		-	-	-	40.00	40.00

For this assay, leaves with 22-25 N4-N5 instar nymphs were collected from the greenhouse insectary. Each treatment was tested in 4 replicates, with each replicate containing 22 nymphs.

Data were analyzed with the software *IBM® SPSS Statistics 26.0* at a 95% Confidence Interval using One-Way ANOVA and Tukey's Honest Significant Difference (HSD) post-hoc test for pairwise comparison. The effect of time on toxicity was assessed through a Repeated Measures One-Way ANOVA. Mortality from control was corrected using Abbott's formula (WHO, 2018):

$$\text{Corrected mortality (\%)} = \frac{(\text{Observed mortality} - \text{Control mortality})}{(100 - \text{Control mortality})} \times 100$$

Where:

$$\text{Observed/Control mortality (\%)} = \frac{\text{Total number of dead nymphs}}{\text{Total sample size}} \times 100$$

For LD₅₀ and LD₉₀ calculation, mortality values without correction were used in a Probit analysis with the software *IBM® SPSS Statistics 26.0* at a 95% Confidence Interval. Probit analysis is a type of regression analysis in which the relationship between the strength of a stimulus and the proportion of cases exhibiting a certain response to this stimulus is measured. This analysis does not need correction of mortality since the test corrects it by itself.

To determine the interaction between the tested compounds, Hewlett and Plackett's model (Don-Pedro, 1996) and Wadley's model (Gisi et al., 1985) were used as per Tak et al. (2016) and Gaire, Scharf, et al. (2020).

Hewlett and Plackett's model is used to determine expected LD₅₀ and LD₉₀ values:

$$E = (a \times LDi(a)) + (b \times LDi(b)) + (c \times LDi(c)) + \dots \dots \dots + (n \times LDi(n))$$

Where *i* refers to the 50 or 90 percent of mortality, *a* is the proportion of compound A in the mixture and LD_{*i*}(*a*) is the LD_{*i*} of compound A, and so on. The interaction ratio was calculated by dividing the expected LD_{*i*} value by the observed LD_{*i*}:

$$\text{Interaction ratio (IR)} = \frac{\text{Hewlett and Plackett's expected LDi of mixture}}{\text{Observed LDi of mixture}}$$

An IR greater than 1.5 indicates a synergistic interaction, a ratio of 1.5 or less and greater than 0.5 indicates an additive interaction and ratios of 0.5 or less indicate antagonism.

3.3.2. Phytotoxicity assay

Evaluation of phytotoxicity was made on six- to- eight- month-old *C. limon* seedlings produced in-house, watered and fertilized frequently as described in Section 3.1.. Three days prior to the start of the assay, each plant was further watered and received 10 mg of slow-release complex granulated blue fertilizer NPK (S) 12-8-16 (20), to assure no nutrient deficiency.

In the assessment of phytotoxicity, the goal was to verify the breaking point in which the testing compounds, at a given concentration and exposure time, began to exhibit phytotoxic effects on the plants. As such, for this assay 40 mL of 12 treatments (4 concentrations (0.80% (2), 1.60% (3), 3.20% (4) and 6.40% (5)) of Eugenol (E), Pulegone (P) and a 1:1 mixture of Eugenol + Pulegone (EP)) were prepared (Table 4).

The preparation of the solutions followed the same serial dilution method applied in Section 3.3.1. Stock solutions for Eugenol and Pulegone in acetone were prepared at a 16% concentration. For volume measurement, a 10 mL graduated glass cylinder was used. Solutions were mixed in a 100 ml glass volumetric flask with the aid of a magnetic stirrer and stored in 20 ml hermetic glass flasks at a temperature of 5±2 °C.

For every treatment, using a mist spray plastic bottle, 5 plants were sprayed thoroughly with each test solution, Negative and Blank Control, until runoff (approximately 6-7 mL) (Figure 10A). Each plant served as a replicate. During all the spray procedures, plants were placed in a dry open environment away from direct sunlight. After spraying, plants were let dry for 1 hour and transferred into the phytoclimatic chamber (*Aralab*® walk-in 13000e) where they were kept at a temperature of 25±2 °C, RH of 70±5% and 14:10 h (L:D).

Phytotoxic measurements were taken before applying the testing compounds and one, two, three, six, and nine days after spraying.

To understand the phytotoxic influence of the tested components, a plant phytotoxicity index (PI) was thus designed. The PI contemplates the recorded info of two factors:

- i) Evolution of leaves effective-chlorophyll-content;
- ii) Visual classification of observed phytotoxicity in each plant at the time of measurement.

Evolution of leaves chlorophyll content was obtained through relative-chlorophyll-content measured on a SPAD-502Plus, *Konica Minolta*[®], Osaka, Japan. The SPAD is a device that allows, in a non-invasive way, to collect and measure the relative chlorophyll content in the leaf at the measurement time. Although the equipment provides only a dimensionless measurement of chlorophyll, such measurement is of great interest in this study as it allows to observe the evolution of the relative content of chlorophyll in the leaf over time.

Table 4 - Prepared concentrations for the phytotoxicity bioassay, with the indication of the code used for each treatment. Volumes used for each compound in the solution are also described.

Solution	Final concentration (%)	Eugenol 16% Solution (mL)	Pulegone 16% Solution (mL)	Acetone (mL)	H ₂ O (mL)	Final volume (mL)
<i>Eugenol</i>						
E2	0.80	2.00	-	22.32	15.68	40.00
E3	1.60	4.00	-	20.64	15.36	40.00
E4	3.20	8.00	-	17.28	14.72	40.00
E5	6.40	16.00	-	10.56	13.44	40.00
<i>Pulegone</i>						
P2	0.80	-	2.00	22.32	15.68	40.00
P3	1.60	-	4.00	20.64	15.36	40.00
P4	3.20	-	8.00	17.28	14.72	40.00
P5	6.40	-	16.00	10.56	13.44	40.00
<i>Eugenol + Pulegone</i>						
EP2	0.80	1.00	1.00	22.32	15.68	40.00
EP3	1.60	2.00	2.00	20.64	15.36	40.00
EP4	3.20	4.00	4.00	17.28	14.72	40.00
EP5	6.40	8.00	8.00	10.56	13.44	40.00
<i>Negative Control</i>						
<i>Blank</i>	-	-	-	24.00	16.00	40.00
	-	-	-	-	40.00	40.00

To determine the evolution of chlorophyll, the first two fully developed leaves were flagged in each plant and measured with the SPAD device every time throughout the whole assay. At each time, measurements were repeated 3 times per leaf and the average chlorophyll content from each leaf and between the two leaves was registered. For a given plant “*x*”, at a given time “*t*”, effective chlorophyll evolution (ECE) was assessed by the difference between the chlorophyll evolution in each plant to the one on the Blank Control:

$$CE_{b,t} - CE_{x,t} = ECE_{x,t}$$

Where “*b*” is the Blank Control and “*CE*” is calculated by the quotient of the difference of the relative chlorophyll “*RC*” measured in the plant at a given time and the relative chlorophyll measured in the same plant at time 0, and the same relative chlorophyll measured at time 0:

$$\frac{(RC_{x,t} - RC_{x,0})}{RC_{x,0}} \times 100 = CE_{x,t}$$

Then, the ECE of each treatment was transformed into a 0 to 10 scale by converting the units, in proportion to the highest measurement.

Visual classification of each plant was also made at every data collection time. This classification was made through the recording of several observable criteria, that represent symptoms of phytotoxicity on the plant: necrotic spots (NS) (discoloration and necrosis of less than 20% of the leaf), necrotic blots (NB) (discoloration and necrosis of more than 20% of the leaf), petiole discoloration (PD), stem discoloration (SD), bud/shoot death (BD) and leaf death (LDth) (Figure 10 B, C and D).

For each criteria a value from 0 to 1 is attributed: NS, NB and petiole PD are relative to the total number of leaves on the plant, while for SD, BD and LDth, only presence (1) or absence (0) is registered. Each of the observed criteria is given a quotation (1x or 2x multiplier) according to their contribution to the phytotoxicity factor. This contribution is based on the severity and possible influence of each criterion on health and normal plant function but also on the perceived commercial value of the plant.

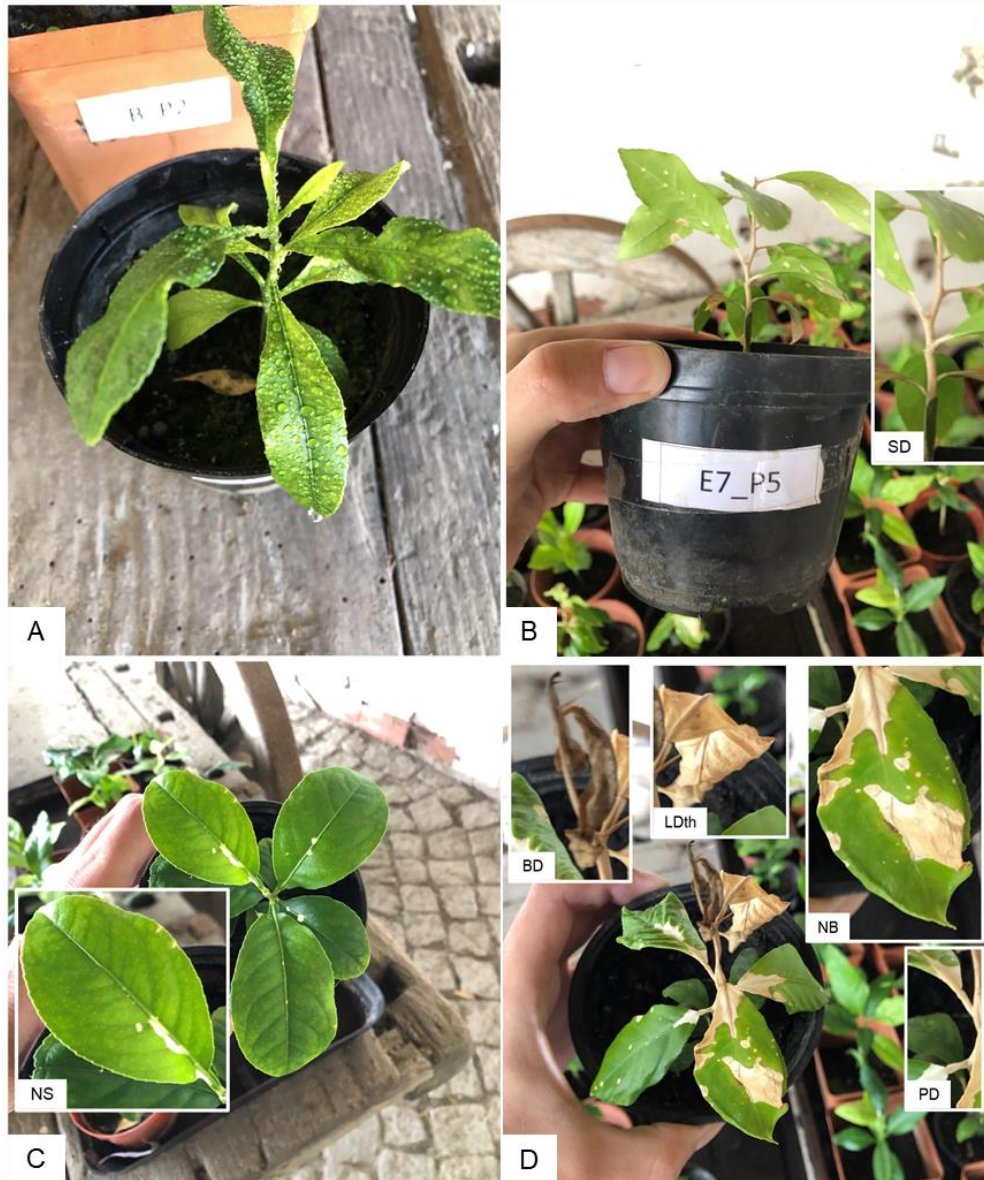


Figure 10 – Example of *C. limon* plant sprayed until runoff (A) and visual classification used to characterize plants phytotoxicity (six days after application): (B) Plant with Stem Discoloration “SD” when treated with E5 (label was not printed correctly); (C) Necrotic Spots “NS” in leaves treated with P9; (D) Observation of bud/shoot Death “BD”, Leaf Death “LDth”, Necrotic Blots “NB” and Petiole Discoloration “PD”, after treatment with E5.

For each plant, at each time, the visual classification “VC” is calculated as follows:

$$NS + PD + BD + 2(NB + SD + LDth) = VC$$

VC is also then transformed to a 0 to 10 scale by converting the units in proportion to the highest observable value.

The final phytotoxicity index (PI) is then calculated:

$$0,4ECE + 0,6VC = PI$$

Phytotoxicity was considered acceptable with a $PI \leq 2,5$. These plants did not seem to exhibit any health problems and loss of normal plant function and perceived commercial value.

Data were analyzed and compared with the software *IBM® SPSS Statistics 26.0* at a 95% Confidence Interval using One-Way ANOVA and Duncan's multiple range test. Finally, the effect of time on phytotoxicity was assessed through a Repeated Measures One-Way ANOVA.

3.3.3. Repellency assay

In the presence of phytotoxic symptoms, it is not possible to establish whether possible changes in landing and oviposition behavior are the result of the repellent effect of the test compounds or the plant's poor health (Hall et al., 2018). As such, Eugenol, Pulegone and the combination of both compounds at 0.80% concentration (E2, P2 and EP2 respectively) were selected for comparison in this assay. The EP3 treatment was also chosen since it did not show phytotoxicity and presented interesting mortality in the toxicity bioassay. The preparation of these four treatments followed the same methodology presented in the preparation of the solutions in Section 3.3.2..

To verify the repellent effect of the test compounds, 6 six- to eight-month-old *C. limon* plants with young shoots were sprayed with each test compound until runoff and allowed to dry for 1 hour. After this period, plants were placed inside 5 L transparent plastic containers with an open top. 16 adults (1:1 sex ratio - at least 4 pregnant females) from the insectary room were captured with the aid of an entomological respirator and transferred into 50 mL Falcon tubes, where they remained for half an hour in order to enhance reproduction. After this, adults were released in each plant and all containers were covered with tulle. All plants were kept at a temperature of 25 ± 2 °C and 70 ± 5 % RH with a photoperiod cycle of 14:10 h (L:D), in the phytoclimatic chamber (Figure 11). To

avoid nutritional deficiencies plants were fertilized with 10 mg of slow-release complex granulated blue fertilizer NPK (S) 12-8-16 (20) 3 days before the test.



Figure 11 – Assay cages together with *C. limon* plants infested with *T. erytreae* adults after spray application of the treatments for the repellency assay (A); Observation of psyllids settled on the shoot of a *C. limon* plant, as well as the possible eggs laid by these adults (B).

At the end of 2 h, 4 h, 8 h, 24 h, 48 h and 72 h, the following elements were registered in each plant:

- i) Number of adults settled in the plant and their sex (settling behavior);
- ii) Number of shoots with eggs (oviposition);
- iii) Number of dead adults.

For analysis of settling behavior and oviposition, One-Way ANOVA was used to analyze differences between the treatments and Fisher's Least Significance Difference (LSD) post-hoc test was utilized for pair-wise comparison. The effect of time on both settling behavior and oviposition was assessed through a Repeated Measures One-Way ANOVA. For the residual mortality, the non-parametric Kruskal-Wallis test was applied for median comparison. All data was analyzed at a 95% Confidence Interval through IBM® SPSS Statistics 26.0.

3.3.4. Ovicidal effect assay

The ovicidal effect of the testing compounds was evaluated by assessing egg viability and observing the development of the nymphs after spray application of each treatment. Per treatment, 5 six-to-eight-month-old *C. limon* plants with young shoots were infested with 10 adults (1:1 sex ratio - 2 pregnant females) of *T. erytreae* from the insectary room, captured with the aid of an entomological aspirator. These adults and plants were kept

in transparent, plastic 5 L containers with a top opening covered with tulle fabric, at a temperature of 25 ± 2 °C and $70\pm 5\%$ RH with a photoperiod cycle of 14:10 h (L:D), in the phytoclimatic chamber.

After spotting eggs in the shoots (approx. 2 to 3 days after infestation), all adults were carefully removed from the containers using an entomological aspirator (Figure 12A). Plants were removed from the recipients and sprayed with the test solutions until runoff, using the same methodologies mentioned in Section 3.3.3..

Similarly to the repellency assay (Section 3.3.3.), 0.80 % concentrations of Eugenol, Pulegone and Eugenol + Pulegone (E2, P2 and EP2, respectively), were selected for this assay. Negative and Blank Control were also tested, and preparation of all solutions followed the same methodology presented in Section 3.3.2. After spray application, plants were left to dry off for 1 hour, and then were returned to the recipient and kept in the phytoclimatic chamber, at the same conditions previously mentioned (Figure 12B). Plants were fertilized with 10 mg of slow-release complex granulated blue fertilizer NPK (S) 12-8-16 (20) 3 days before infestation and watered daily throughout all of the assay.



Figure 12 - Shoots of a *C. limon* plant with eggs of *T. erytreae*, after removal of adults and prior to the treatments spray application (A); *C. limon* plants with eggs kept inside plastic containers for posterior observation of the Hatch Ratio and the Instar Development Time (B).

At the time of spray, the number of shoots with eggs was registered. Development of the eggs was then followed during the next 18 days after spray - daily during the first 7 days, and at the 10th, 14th and 18th day. In each observation was registered:

- i) Number of shoots with eggs and nymphs;
- ii) Nymphs' development stages (N1, N2, N3, N4, N5 instars);
- iii) Number of days passed after egg posture.

Data were analyzed for the:

- i) Hatch ratio (HR): the ratio between the initial number of shoots with eggs and the number of shoots with 1st instar (N1) nymphs (before N2 instar development was registered), given by:

$$\frac{\text{Shoots with N1 nymphs}}{\text{Initial shoots with eggs}} \times 100 = HR$$

- ii) Instar Development Time (IDT): Analysis of instar development, at a given time after posture (5, 6, 7, 10, 14, 18 days)

Since two of the ANOVA assumptions were not met for both HR and IDT data analysis (population was not normally distributed and population's variances were not homogeneous), treatments were compared by applying a Kruskal-Wallis non-parametric test at a 95% Confidence Interval. IBM® SPSS Statistics 26.0 was used for data analysis.

3.3.5. Semi-Field assay

To further establish the practical potential of utilizing these compounds for *T. erytreae* control, as well as better understand the influence in reducing the application dosages of these compounds, a semi-field assay was carried out. Since this was the last assay to be performed, previous results were accounted for when choosing the treatment to be tested: in concentrations that did not pose a significant phytotoxic effect on *C. limon*, the combination of both active compounds exhibited the highest nymphal mortality, as such, the Eugenol and Pulegone mixture at 1.60% concentration (EP3) was selected for this assay. The preparation of the solution followed the same methodology employed in Section 3.3.2..

Initially, this assay was to be performed on 35 two- to three-year-old *C. limon* plants, which were purposely kept in the greenhouse and infested with *T. erytreae* adults one month prior to the start of the assay. However, due to the release of *T. dryi* in the vicinity of Campus Agrário de Vairão, by the time the assay was to be started, a high parasitism rate (>80%) was observed in greenhouse nymphs that were to be used, precluding the proper assessment of the effectivity of the testing solution. As such, 30 two- to three-year-old *C. limon* plants, free of *T. dryi*, were ordered and infested with *T. erytreae* adults inside entomological cages, however, since these plants were previously treated with insecticides in the nursery house, the establishment of the *T. erytreae* adults in these plants and the further completion of its life cycle was not possible, again preventing the completion of the assay in its initial terms. These limitations were overcome in the

following months by infesting 20 six- to eight-month-old *C. limon* plants with *T. erytreae* adults to induce oviposition in these plants, applying the same methodology employed in Section 3.3.4.. From these, only six plants presented N4 and N5 instar nymphs, and were thus utilized for this test.

The experiment was then performed through spray application of the EP3 solution using a mist spray plastic bottle (Figure 13B). Prior to the application, in each plant, a Syngenta® 30 x 30 mm water sensitive paper was secured on the underside of one randomly selected leaf (Figure 13A). Additionally, to assure compatibility in comparing the results of this assay with the toxicity bioassay (Section 3.3.1) and ease the count of the number of dead nymphs, only N4 and N5 instar nymphs were maintained in the plants, while the rest were removed with the aid of a straight fine dissection needle. Volumes were measured using a 10 mL graduated glass cylinder. After spray application, a photograph of each water sensitive paper was taken for further analyzing the spray coverage area (SCA) (Figure 13C). Plants were kept inside an entomological cage in the outside field of Campus Agrário de Vairão, exposed to the environmental conditions.

The testing solution was applied in two different volumes:

- i) V1: 6.4 mL, the corresponding volume of spray application until runoff (calculated by the average volume of runoff on five applications);
- ii) V2: 1.6 mL, a fourth of V1 volume, selected by reducing the V1 volume until the SCA on the water sensitive paper was reduced to about half of the observed area on V1.

For each treatment (V1 and V2) three *C. limon* plants were used as replicates. The number of *T. erytreae* nymphs in each plant was registered before the spray application, as well as after 24 and 48 h with the aid of a head magnifying glass. Nymphs were considered dead when no movement was observed for 30 seconds when probed and rotated with a straight fine dissection needle.



Figure 13 - Application procedure of the testing solution through spray application on *C. limon*. Water sensitive paper located in the underside of the leaf (A); Spray application with the aid of a mist spray plastic bottle (B); Photograph of the water sensitive paper after spray application, evidencing with the blue coloration the droplets dispersal and coverage area (C).

To determine the effect of these compounds on the nymphal populations in the plants, the percentual reduction of the number of nymphs (Nymphal Reduction (%)) was calculated for each plant in each observation time “*t*” (24 or 48 hours) by:

$$100 - \frac{(\text{Number of Nymphs}_t - \text{Number of Dead Nymphs}_t) \times 100}{\text{Initial Number of Nymphs}} = \text{Nymphal R. (\%)}$$

To determine the SCA, a photograph of each water sensitive paper was taken after application and the image was then processed through *SnapCard*® software (Version 2.1, iOS 14.7.1), Department of Primary Industries and Regional Development, Government of Western Australia.

Data for the Nymphal Reduction (%) between both application volumes was analyzed and compared in a One-Way ANOVA, independently for each observation time. The effect of time on the Nymphal Reduction (%) was assessed through a repeated Measures One-Way ANOVA. All data was analyzed at a 95% Confidence Interval using the software *IBM*® *SPSS Statistics 26.0*.

4. Results

4.1. Preliminary assays

4.1.1. Selection of dilution solvents

The proper selection of a solvent for this work was essential. Apart from correctly and thoroughly dissolve the testing compounds at the chosen concentrations, the selected solvent couldn't by itself exert more than 20% mortality on the testing subjects.

S1, S2 and S3 were the first prepared and applied solutions. After 24 h S1 and S2 solutions had the highest Mortality Rate (MR) (\bar{x} =64.75%; s =26.75% and \bar{x} =74.43%; s =8.75% respectively) while S3 presented the lowest (\bar{x} =2.63%; s =3.04%) (Figure 14). Given that the only differencing factor between these three solutions was the usage of *Tween*[®] 80 in S1 and S2, there seems to be a relation between the observed mortality and this emulsifier. S3 was then used to dissolve Eugenol to 6.40% concentration but when adding water to complete the solution, drops of oil began to form in the solution.

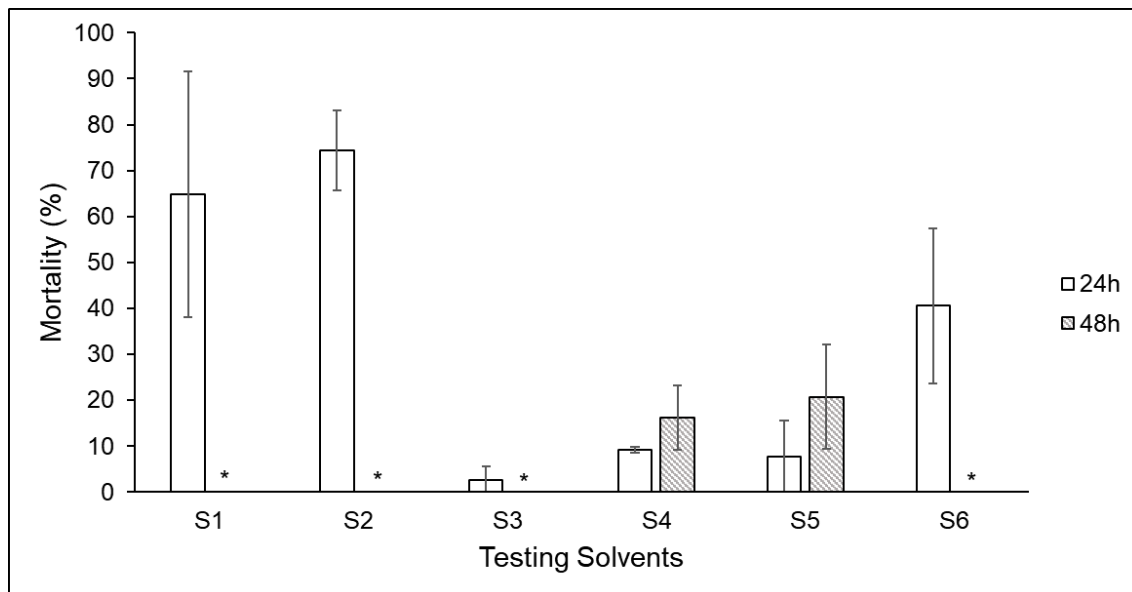


Figure 14 - Nymph mortality rate ($n=4$) (\pm SD) for each testing solvent (S1, S2, S3, S4, S5 and S6), after 24 and 48 h of topical application. * - not tested for 48 h mortality.

Three more solutions were then prepared. S6 with 100% acetone dissolved the oil but had high MR after 24 h (\bar{x} =40.56%; s =16.86%), while the S5 solution dissolved the oil and had a low MR after 24 h (\bar{x} =7.78%; s =7.87%). Due to the registered low mortality after 24 h, the same solution was observed after 48 h. However, at 48 h, mortality was higher than 20%, with high deviation (\bar{x} =20.72%; s =11.38%). S4 was then prepared with

60% acetone. Less MR was registered both after 24 h (\bar{x} =9.12%; s =0.62%) and 48 h (\bar{x} =16.23%; s =7.02%) (Figure 14). The same solution was clear and homogenous when preparing Eugenol and Pulegone at 6.40% concentrations, indicating that acetone at 60% could be utilized for dissolving the testing compounds without potentially compromising the toxicity evaluations.

4.1.2. Verifying differences in susceptibility between populations

To verify possible differences in susceptibility between Insectary Nymphs (IN) and Greenhouse Nymphs (GN), an assessment of their toxicity to various testing solvents (S1, S2 and S3) was made through topical application.

When applying S1, it was noted that IN were less susceptible to the solvent (\bar{x} =41.99%; s =7.96%), than GN (\bar{x} =87.50%; s =3.54%). The S2 solution however showed no notable differences between both IN (\bar{x} =76.19%; s =13.47%) and GN populations (\bar{x} =72.67%; s =6.01%). As for the S3 solution, lower MR was noted on the GN (\bar{x} =0%; s =0%) when comparing to the IN (\bar{x} =2.63%; s =3.72%), albeit by a very small difference.

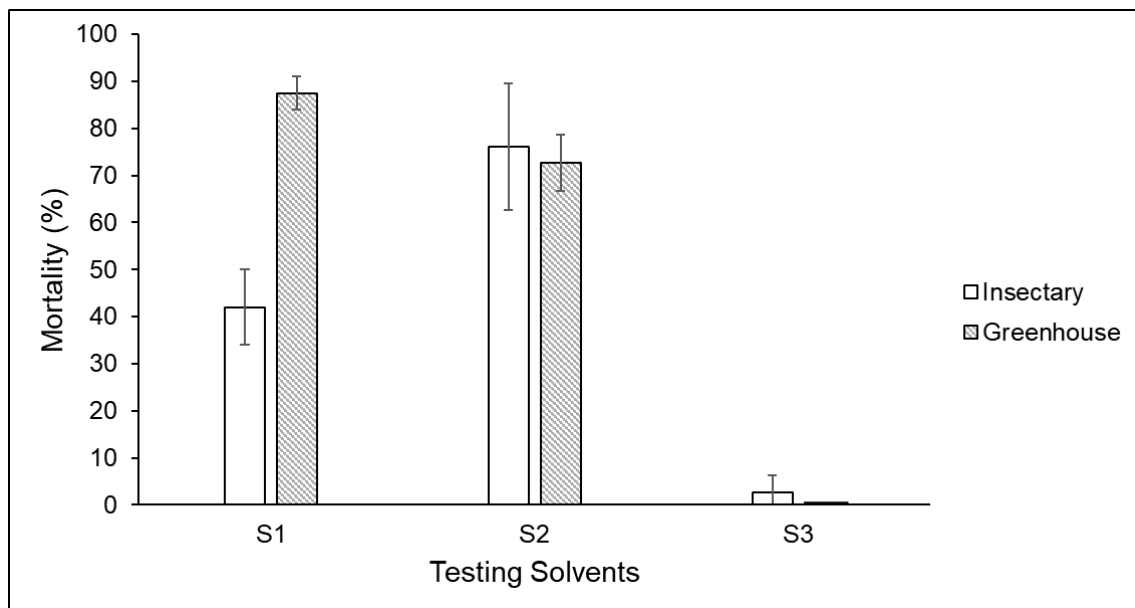


Figure 15 - Mortality rate comparison ($n=2$) (\pm SD) between Insectary and greenhouse nymphs for three testing solvents (S1, S2 and S3) after 24 h of topical application.

The results from the application of S1 contraries the rest of the observed results but such difference may be due to incorrect application of the solution on nymphs' dorsal regions. The application of the S1 solution on IN constituted one of the first trials in the very beginning of this work, when the methodology was still being experimented. Regardless,

when observing the toxicity of S2 and S3 it was also noted that GN may be slightly more resistant to the test solvents, an expected outcome due to ideal development conditions in the insectary, especially when using only acetone as the dissolvent.

Since the mean values of mortality between both populations are within the standard deviation range, these preliminary results show that IN and GN may both be used for the toxicity bioassay. Nonetheless, since GN were slightly less susceptible to the action of the tested solvents, this population should be used in the toxicity bioassay for better interpretation of the final results.

4.2. Toxicity bioassay

The mortality of testing treatments (Table 3) in *T. erytreae* nymphs was evaluated using a topical contact assay and the number of dead insects was counted at the end of 24 h and 48 h. Statistical analysis of the toxic effects of these treatments was done separately for the two observations using a One-Way ANOVA and results were compared between the different treatments applied, as well as the 0.06% *Confidor*[®] Positive Control, with Tukey's Honest Significant Difference (HSD) post-hoc test. Repeated measures One-Way ANOVA was used for an evaluation of the effect of time on toxicity. The data were also subjected to a Probit regression analysis, allowing the determination of these compounds' LD₅₀ and LD₉₀ values. The obtained LD₅₀ and LD₉₀ values were then compared between the compounds Eugenol and Pulegone, and their joint solution Eugenol+Pulegone, thus also allowing the assessment of their potentiator effect when combined: antagonism, additive effect or synergism.

One-Way ANOVA revealed that the mortality on the tested N4-N5 instar nymphs was different between treatments, both at the end of 24 h ($F(15,48)=79.144$; $p<0.001$) and at the end of 48 h ($F(15,48)=34.865$; $p<0.001$).

Through the repeated measures one-way ANOVA, it was possible to verify that mortality was also significantly different between the 24 and 48 h observations Wilks' Lambda=0.244, $F(1,48)=148.776$, $p<0.01$, with an observed increment in mortality (Figure 16 and 17), evidencing that time increased the toxicity of the tested compounds on the testing subjects.

At the end of 24 h, it was possible to notice that the effect on mortality was dependent on the concentration applied for all solutions tested, increasing for each increment in concentration although not always significantly. In the case of Eugenol, there was a significant increase in mortality rate when the concentration was increased from

treatment E3 ($\bar{x}=35.79$, $s=8.56$) to E4 ($\bar{x}=65.81$, $s=7.66$) ($p<0.01$) and from E4 to E5 ($\bar{x}=95.11$, $s=3.38$) ($p<0.01$). As for Pulegone, the only significant increase was verified between P3 ($\bar{x}=44.39$, $s=5.71$) and P4 ($\bar{x}=82.91$, $s=4.51$) ($p<0.01$). For the combination of the two active compounds, Eugenol + Pulegone continued with a mortality of less than 20% up to the 0.80% concentration. It was in the transition from EP2 ($\bar{x}=15.24$, $s=8.08$) to EP3 ($\bar{x}=51.02$, $s=8.15$) that the biggest significant rise in mortality was observed ($p<0.01$), significantly increasing again when the concentration was raised from EP3 to EP4 ($\bar{x}=83.74$, $s=7.40$) ($p<0.01$). No significant differences were observed when increasing the concentrations from 0.40% and 0.80% for either Eugenol, Pulegone and their mixture ($p>0.05$) (Figure 16).

When comparing the mortality effects of the different treatments within the same concentrations, no treatment significantly differed from each other when applied at the same concentration ($p>0.05$). However, when comparing different treatments between 1.60%, 3.20% and 6.40% concentrations, it can be observed that applying Pulegone or Eugenol + Pulegone at double the concentration of Eugenol, significantly increased the mortality, whereas no significant difference was noted in utilizing Eugenol at double the concentration of either Pulegone or Eugenol + Pulegone: significant differences were observed between P5/EP5 ($\bar{x}=98.75$, $s=2.17$; $\bar{x}=100$, $s=0$, respectively) and E4 ($\bar{x}=65.81$, $s=7.66$) ($p<0.01$), as well as between EP4 ($\bar{x}=83.74$, $s=7.40$) and E3 ($\bar{x}=35.79$, $s=8.56$) ($p<0.01$); but no significant differences were observed between using E5 and P4/EP4 ($p=0.646$; $p=0.747$ respectively) and between E4 and EP3 ($p=0.343$) (Figure 16).

At the end of 24 h the highest mortality rate (over 80%) was observed for treatments P4, EP4, E5, P5 and EP5. Additionally, these compounds did not show any significant differences between themselves as well as with the Positive Control (C+) ($p>0.05$) (Figure 16).

On the other hand, treatments EP1, EP2, E1, P1, E2 and P2 produced the lowest mortality in the test subjects (under 30%). Apart from EP1 ($\bar{x}=8.45$, $s=3.74$), which had a significantly lower toxicity than P2 ($\bar{x}=28.73$, $s=8.93$) ($p<0.05$), no significant differences were noted between these treatments ($p>0.05$) (Figure 16).

Treatments E3, P3, EP3 and E4 showed intermediate mortality, between 30 and 70%.

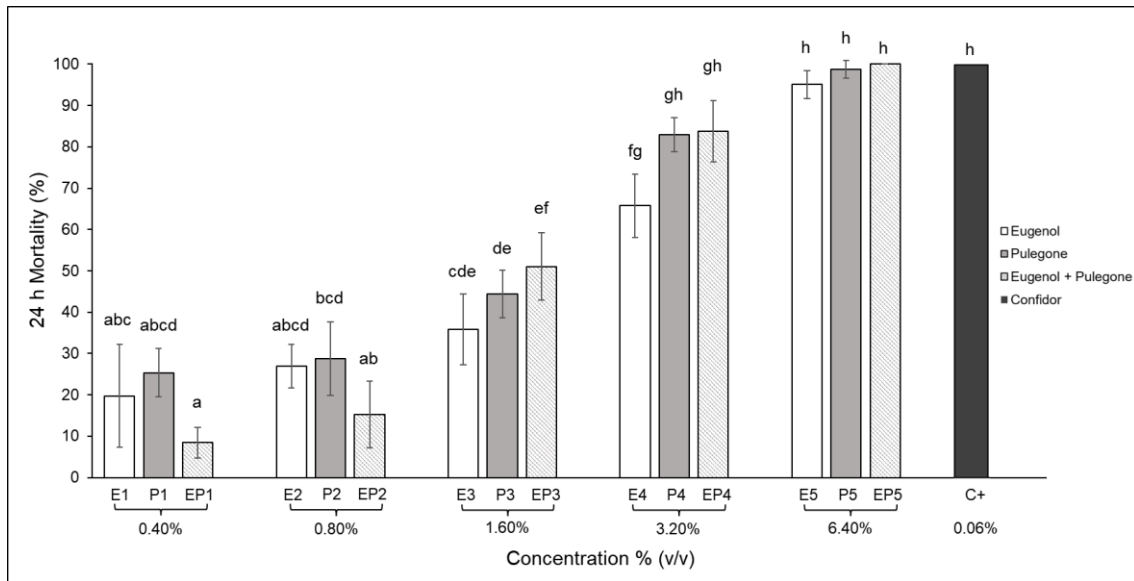


Figure 16 - Mean (\pm SD) ($n=4$) mortality rate of *T. erytreae* nymphs when treated through topical application with the different treatments (Eugenol (E), Pulegone (P) and Eugenol+Pulegone (EP)) at different concentrations (v/v %) (0.40% (1), 0.80% (2), 1.60% (3), 3.20% (4), 6.40% (5)) and the Confidor Positive Control (C+ at 0.06%), at the end of 24 h. Different letters represent significant differences between treatments, as per Tukey HSD ($p<0.05$).

At the end of 48 h, the positive relationship between the increase in the concentration of different treatments and the increase in mortality was also noted. The increase in the concentration of Eugenol translated into a significant increase in mortality over the E3 ($\bar{x}=57.24$, $s=13.10$) and E4 ($\bar{x}=84.50$, $s=7.98$) ($p<0.05$) treatments. For Pulegone, it already displayed relatively high mortality in the P1 and P2 concentrations but only rose significantly when increased from P3 ($\bar{x}=57.50$, $s=11.62$) to P4 ($\bar{x}=95.93$, $s=4.50$) ($p<0.01$). The mortality of the combination of the two Eugenol+Pulegone compounds varied greatly as concentrations were increased, raising its mortality significantly when increased from EP2 ($\bar{x}=35.72$, $s=8.97$) to EP3 ($\bar{x}=80.06$, $s=4.61$) ($p<0.01$) (Figure 17).

When comparing the mortality rate between the different treatments in each concentration, no significant differences were again noted for all concentrations. However, and similarly to the observed at the 24 h period, some treatments at double the concentration did not produce significantly more mortality when compared to others that were at half the concentration. This time, it was possible to observe this occurrence at lower concentrations than before (0.80%, 1.60%, 3.20%), between Eugenol/Pulegone and their combined oil. In these concentrations, the combination of both compounds produced significantly more mortality when at double the concentration than the individual compounds, but not the other way around: EP3 ($\bar{x}=80.06$, $s=4.61$) was significantly more toxic to the test subjects than E2 ($\bar{x}=40.31$, $s=5.20$) ($p<0.01$), and EP4

(\bar{x} =90.50, s =4.50) was also significantly more toxic than E3 (\bar{x} =57.24, s =13.10) (p <0.01) and P3 (\bar{x} =57.50, s =11.62) (p <0.01); but no significant differences were noted between E3 and EP2 and between E4/P4 and EP3 (p >0.05) (Figure 17).

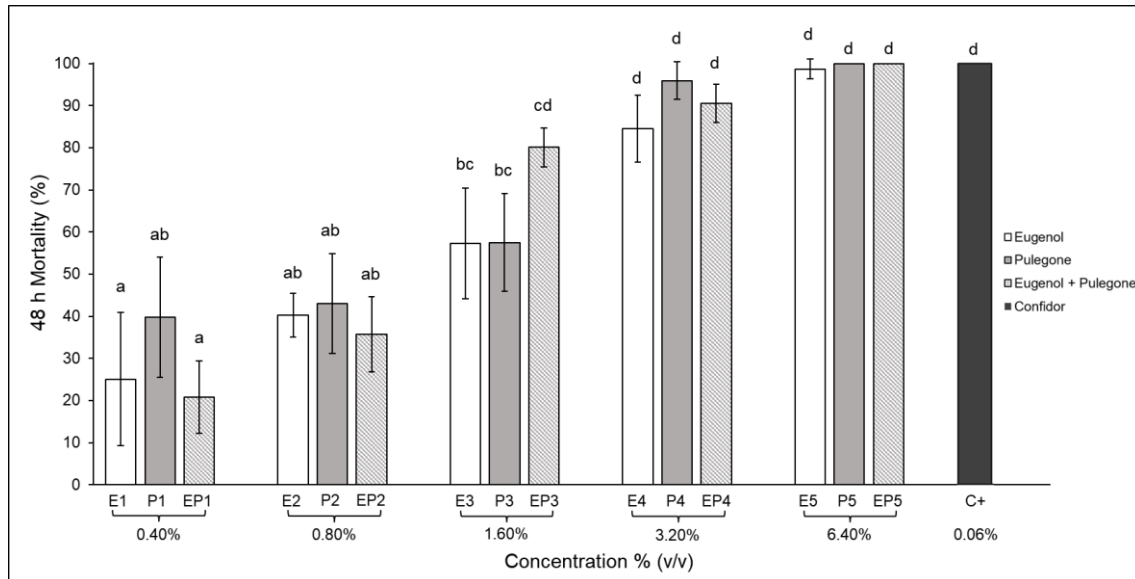


Figure 17 - Mean (\pm SD) (n =4) mortality rate of *T. erytreae* nymphs when treated through topical application with the different treatments (Eugenol (E), Pulegone (P) and Eugenol+Pulegone (EP)) at different concentrations (v/v %) (0.40% (1), 0.80% (2), 1.60% (3), 3.20% (4), 6.40% (5)) and the Confidor Positive Control (C+ at 0.06%), at the end of 48 h. Different letters represent significant differences between treatments, as per Tukey HSD (p <0.05).

When cumulative mortality was observed at the end of 48 h, increases in the effects of these compounds on nymph mortality were noted. In this period, treatments EP3, E4, EP4, P4, E5, P5 and EP5 showed very high mortality, above 80%. Comparatively with the first 24 h observation, EP3 and E4 now join P4, EP4, E5, P5 and EP5 in the treatment groups with no significant differences with the Positive Control (C+) (p >0.05). Regarding the treatments that showed lower mortality, this time, none of them had a mortality lower than 20%, with the lowest mortality values attributed to treatments EP1 (\bar{x} =20.79) and E1 (\bar{x} =25.04). The remaining treatments EP2, P1, E2, P2, E3 and P3 now showed average mortality between 36 and 57% and were not significantly different from each other (p >0.05) (Figure 17).

In terms of lethal dosages, both at the end of 24 h and 48 h, the oil with the lowest LD₅₀ was Pulegone (P) (0.005 and 0.003 μ L.nymph⁻¹, respectively). As for the LD₉₀, the combination of the two oils Eugenol+Pulegone (EP) presented the lowest value, both at the end of 24 h (LD₉₀=0.020 μ L.nymph⁻¹) and at the end of 48 h (LD₉₀=0.012 μ L.nymph⁻¹). Of the three solutions evaluated, Eugenol (E) was consistently the least toxic to the

nymphs in both tested periods, both in the evaluation of LD₅₀ (24h=0.007; 48h=0.004 μL.nymph⁻¹) and LD₉₀ (24h=0.040; 48h=0.012 μL.nymph⁻¹) (Table 5).

The combination of the two oils was beneficial in both mortality and time evaluations, always presenting an additive effect (Ratio LD₅₀, LD₉₀>0.5). In fact, in the evaluation of mortality at the end of 24 h, the solution EP demonstrated that the joining of the two oils revealed a synergistic effect in their interaction to reduce the population by 90% (ratio LD₉₀ 1.652>1.5) even though at the end of 48 h this same observation only indicated an additive effect (ratio LD₉₀=1.366) (Table 5).

Table 5 – Insecticidal activities of Eugenol (E), Pulegone (P) and Eugenol+Pulegone (EP) to fourth to fifth instar *T. erytreae* nymphs by topical application, at 24 and 48 h observations, with the respective indication of Fiducial Limits. Ratio of interaction is also described in the table. At bold, the lowest observed LD₅₀ and LD₉₀ between treatments is evidenced, as well as the observation of synergism.

24 h

Treatment	N	LD ₅₀ (μL.nymph ⁻¹)	(Fiducial limits)	LD ₉₀ (μL.nymph ⁻¹)	(Fiducial limits)	Ratio LD ₅₀ (interaction)	Ratio LD ₉₀ (interaction)
E	446	0.007	(0.005 – 0.009)	0.040	(0.026 – 0.080)	-	-
P	440	0.005	(0.004 – 0.006)	0.024	(0.019 – 0.035)	-	-
EP	442	0.006	(0.006 – 0.007)	0.020	(0.016 – 0.025)	0.954 (additive)	1.652 (synergistic)

48 h

Treatment	N	LD ₅₀ (μL.nymph ⁻¹)	(Fiducial limits)	LD ₉₀ (μL.nymph ⁻¹)	(Fiducial limits)	Ratio LD ₅₀ (interaction)	Ratio LD ₉₀ (interaction)
E	446	0.004	(0.003 – 0.004)	0.019	(0.015 – 0.027)	-	-
P	440	0.003	(0.002 – 0.004)	0.015	(0.010 – 0.027)	-	-
EP	442	0.004	(0.003 – 0.004)	0.012	(0.010 – 0.016)	0.952 (additive)	1.366 (additive)

4.3. Phytotoxicity assay

Phytotoxicity of the testing treatments was evaluated on six- to eight-month-old *C. limon* potted plants and registered after 24 h, 48 h, 72 h, 6 days and 9 days of spray application. Statistical analysis of phytotoxicity was made separately for all the different observations and results were compared between treatments. Such comparison was only carried out after verifying no differences between Negative and Blank Control plants for the whole

testing period. Repeated measures One-Way ANOVA was used for an evaluation of the effect of time on phytotoxicity. The assessment of phytotoxicity was made using a phytotoxicity index (PI) designed for this purpose. This index comprises the combined information of the plants visual classification (VC) with the evolution of chlorophyll content (ECE).

Treatments were compared in One-Way ANOVA, revealing the existence of significant differences in phytotoxicity between them, for all testing periods: 24 h ($F(11,48)=9.370$; $p<0.01$); 48 h ($F(11,48)=12.350$; $p<0.01$); 72 h ($F(11,48)=12.107$; $p<0.01$); 6 days ($F(11,48)=14.618$; $p<0.01$); 12 days ($F(11,48)=25.175$; $p<0.01$).

Through the repeated measures one-way ANOVA, it was possible to verify that time does influence the phytotoxicity level, Wilks' Lambda=0.095, $F(4,45)=106.777$, $p<0.01$. Furthermore, there was also a significant difference when pairwise comparing each observation period ($p<0.05$), with an increment in phytotoxicity, suggesting that time increased the phytotoxicity in the *C. limon* plants. As such, results were compared always considering the phytotoxicity evolution throughout the period of the assay.

When comparing treatments within the same concentration using Duncan's post-hoc Multiple Range Test (DMRT), it is possible to verify that in all treatments at the 0.80% concentration E2, P2, EP2 phytotoxicity did not differ significantly in all observed periods ($p>0.05$).

However, with an increase in concentration, differences between treatments are exacerbated. For treatments at a concentration of 1.60% (E3, P3, EP3), while no significant difference was noted between them during the first 6 days ($p>0.05$), significant differences in phytotoxicity were noticed after 9 days, where the combination of both oils EP3 ($\bar{x}=1.20$; $SE=0.24$) presented a phytotoxicity index significantly lower than that of Eugenol E3 ($\bar{x}=3.07$; $SE=0.93$) ($p<0.05$) (Table 6).

At a concentration of 3.20%, differences in phytotoxicity between treatments started to be noted earlier: at the end of 72h, E4 ($\bar{x}=3.27$; $SE=0.37$) presents a phytotoxicity index significantly higher than that of EP4 ($\bar{x}=1.44$; $SE=0.12$) ($p<0.05$) and Pulegone P4 ($\bar{x}=1.67$; $SE=0.24$) ($p<0.05$). At the end of 6 days the same compound E4 ($\bar{x}=4.16$; $SE=0.30$) only presents a phytotoxicity significantly higher than P4 ($\bar{x}=2.06$; $SE=0.20$) ($p<0.05$), whereas at the end of 9 days, and similarly to the 6-day observation, the phytotoxicity index of E4 ($\bar{x}=5.38$; $SE=0.17$) is again significantly higher than P4 ($\bar{x}=2.61$; $SE=0.18$) ($p<0.05$) and EP4 ($\bar{x}=3.62$; $SE=0.40$) ($p<0.05$) (Table 6).

For the highest concentration of 6.40%, differences between the testing compounds started to be detected at the first observation. After 24 h, E5 ($\bar{x}=3.26$; $SE=0.76$) presents

significantly higher phytotoxicity than that of EP5 ($\bar{x}=2.05$; $SE=0.32$) ($p<0.05$), which in turn is also significantly higher than P5 ($\bar{x}=1.11$; $SE=0.18$) ($p<0.05$); From 48 hours onwards, the phytotoxicity of the P5 treatment was significantly lower than that of the EP5 and E5 treatments ($p<0.05$), with the only exception at the 6th-day observation, when no significant differences between treatments were noted ($p>0.05$) (Table 6).

In this work, a $PI<2.5$ was also defined as an acceptable phytotoxicity limit. At the lowest concentration (0.80%), all treatments (P2, EP2 and E2) presented an index below the established limit. At a concentration of 1.60%, after 9 days, only E3 showed too much phytotoxicity ($\bar{x}=3.07$). At higher concentrations (3.20% and 6.40%), all tested compounds obtained a $PI>2.5$ at the end of the assay. At these high concentration levels, Eugenol was also the compound exhibiting higher phytotoxicity. Furthermore, it was the compound at which treated plants consistently first exhibited high phytotoxicity (48 h for E4; 24 h for E5), followed by the combination of Eugenol + Pulegone (6 days for EP4; 48 h for EP5) and Pulegone (9 days for P4; 6 days for P5) (Table 6).

Table 6 - Mean and standard error (SE) of the phytotoxicity index ($n=5$) registered for each treatment after 24, 48 and 72 hours and 6 and 9 days of application in *C. limon*. Post-hoc analysis through Duncan was made independently for each time. Different letters above means represent significant differences between treatments as per Duncan multiple range test ($p<0.05$). Means with $PI \geq 2.5$ represented at bold.

Treatment	Phytotoxicity Index (0-10) \pm SE				
	24 h	48 h	72 h	6 days	9 days
0.80%					
E2	0.33 ^a \pm 0.11	0.62 ^{ab} \pm 0.09	0.87 ^a \pm 0.09	1.24 ^a \pm 0.10	1.72 ^{ab} \pm 0.13
P2	0.28 ^a \pm 0.09	0.43 ^a \pm 0.06	0.49 ^a \pm 0.04	0.95 ^a \pm 0.06	1.46 ^a \pm 0.13
EP2	0.27 ^a \pm 0.06	0.44 ^a \pm 0.09	0.58 ^a \pm 0.14	1.00 ^a \pm 0.27	1.33 ^a \pm 0.34
1.60%					
E3	0.19 ^a \pm 0.07	0.53 ^a \pm 0.18	1.03 ^a \pm 0.30	2.03 ^{ab} \pm 0.84	3.07^{bc} \pm 0.93
P3	0.82 ^a \pm 0.20	1.08 ^{ab} \pm 0.28	1.27 ^a \pm 0.33	1.90 ^{ab} \pm 0.31	2.44 ^{abc} \pm 0.22
EP3	0.14 ^a \pm 0.04	0.13 ^a \pm 0.07	0.46 ^a \pm 0.12	1.07 ^a \pm 0.19	1.20 ^a \pm 0.24
3.20%					
E4	1.91 ^{bc} \pm 0.35	2.78^{cd} \pm 0.38	3.27^{cd} \pm 0.37	4.16^{cd} \pm 0.30	5.38^d \pm 0.17
P4	0.57 ^a \pm 0.14	0.98 ^{ab} \pm 0.20	1.67 ^{ab} \pm 0.24	2.06 ^{ab} \pm 0.20	2.61^{abc} \pm 0.18
EP4	0.99 ^{ab} \pm 0.14	1.19 ^{ab} \pm 0.12	1.44 ^{ab} \pm 0.12	2.97^{bc} \pm 0.37	3.62^c \pm 0.40
6.40%					
E5	3.26^d \pm 0.76	4.52^e \pm 0.74	5.00^e \pm 0.62	5.94^e \pm 0.70	7.51^e \pm 0.47
P5	1.11 ^{ab} \pm 0.18	1.88 ^{bc} \pm 0.40	2.67^{bc} \pm 0.80	4.97^{de} \pm 0.47	5.72^d \pm 0.28
EP5	2.05 ^c \pm 0.32	3.90^{de} \pm 0.77	4.24^{de} \pm 0.63	6.35^e \pm 0.81	8.23^e \pm 0.81

At the final observation, differences between the various treatments are more apparent, permitting the direct comparison of the phytotoxic effects produced in *C. limon* plants treated with different concentrations of Eugenol, Pulegone and Eugenol+Pulegone. From all the tested solutions, EP5 ($\bar{x}=8.23$; $SE=0.81$) and E5 ($\bar{x}=7.51$; $SE=0.47$) were the most toxic. P5 ($\bar{x}=5.72$; $SE=0.28$), although at the same concentration of the most toxic solutions, presented a significantly lower phytotoxicity, with no significant differences than E4 ($\bar{x}=5.38$; $SE=0.17$), a treatment at half the concentration. Compared with E4, treatments EP4 ($\bar{x}=3.62$; $SE=0.40$), E3 ($\bar{x}=3.07$; $SE=0.93$) and P4 ($\bar{x}=2.61$; $SE=0.18$) presented significantly lower phytotoxicity, but still too high to be considered acceptable ($PI>2.5$). Plants showed less phytotoxicity when sprayed with treatments P3 ($\bar{x}=2.44$; $SE=0.22$), E2 ($\bar{x}=1.72$; $SE=0.13$), P2 ($\bar{x}=1.46$; $SE=0.13$), EP2 ($\bar{x}=1.33$; $SE=0.34$) and EP3 ($\bar{x}=1.20$; $SE=0.24$), all while simultaneously preserving the acceptable limit for phytotoxicity ($P<2.5$) (Table 6).

4.4. Repellency assay

Following the results from the phytotoxic assay and based on the existence of acceptable phytotoxicity on the treated plants, 4 treatments were selected for repellency analysis: E2, P2, EP2 and EP3.

To evaluate the repellent potential of the selected treatments, observations of the settling behavior of 16 *T. erytreae* adults (8 males and 8 females) were performed 2 h, 4 h, 8 h, 24 h and 72 h after spray and let dry. These insects were released on 6 plants per treatment; however, one outlier was observed for every treatment and control groups. These were removed from the data analysis which ended up comparing 5 replicates per treatment. In the same period, the number of shoots with eggs was registered to evaluate the oviposition deterrent potential of the compounds.

For the settling behavior and oviposition assays, One-Way ANOVA was used to compare differences between the treatments and Fisher's Least Significance Difference (LSD) post-hoc was utilized to perform a pair-wise comparison. For the residual mortality effect, the non-parametric Kruskal-Wallis test was applied for median comparison. Possible residual effect mortality was also accounted for during all of the experiments. For the settling behavior and oviposition observations, repeated measures One-Way ANOVA was used for an evaluation of the effect of time on these variables.

For settling behavior, One-Way ANOVA indicated that there was no significant differences between treatments at the 2 h observation ($F(5,24)=2.102$; $p=0.100$) and at

the 72 h observation ($F(5,24)=1.981$; $p=0.118$); significant differences were observed after 4 h ($F(5,24)=3.68$; $p<0.05$), 8 h ($F(5,24)=3.731$; $p<0.05$), 24 h ($F(5,24)=5.030$; $p<0.05$) and 48 h ($F(5,24)=1.981$; $p<0.05$).

Through the repeated measures one-way ANOVA, it was possible to verify that time did influence the settling behavior, Wilks' Lambda=0.339, $F(5,20)=7.803$, $p<0.01$. Furthermore, the pairwise comparison evidenced the alterations in settling behavior through time, where a significant increase was noted between the 2 h observation and the 4 h observation ($p<0.05$), after which psyllids did not significantly alter their settling behavior until the end of the assay ($p>0.05$). It was though noted that after 48 h, the number of settled psyllids decreased and were once again not significantly different than the ones first observed at the first 2 h observation ($p<0.05$). This analysis does suggest that time influences the settling behavior, with an increment in the number of psyllids after 4 h, which after 48 h decreased their numbers in the plants to values similar to the ones noted on the first 2 h observation.

During all of the observations for settling, no significant differences between Blank and Negative Control plants were noted ($p>0.05$). No significant differences were noted between psyllids settled on plants treated with the EP2 and P2 compounds and Negative Control plants ($p>0.05$) (Figure 18). Additionally, the number of males vs females settled was not significantly different for all treatments, at all times ($p>0.05$).

As mentioned in the One-Way ANOVA analysis, no differences between treatments were observed at the 2 h observation ($p>0.05$). Afterwards, the number of settled psyllids rose consistently for the following 4 h (LSD=1.818), 8 h (LSD=1.826), and 24 h (LSD=1.556) observations. During all of this period, the settling behavior was constant between treatments with consistently and significantly fewer psyllids settled on plants treated with the EP3 ($\bar{x}_{4h}=7.67$; $\bar{x}_{8h}=7.83$; $\bar{x}_{24h}=8.33$) and E2 ($\bar{x}_{4h}=8.40$; $\bar{x}_{8h}=8.60$; $\bar{x}_{24h}=8.40$) when compared to the number of psyllids settled on the Blank ($\bar{x}_{4h}=13.40$; $\bar{x}_{8h}=14.40$; $\bar{x}_{24h}=14.40$) and Negative Control ones ($\bar{x}_{4h}=13.00$; $\bar{x}_{8h}=13.40$; $\bar{x}_{24h}=14.00$) ($p<0.05$). Additionally, presumably due to psyllids trying to escape the enclosed space, the results for the following observations should account for the possible inability of the caught-up insects to return to the plant. Nonetheless, after 48 h (LSD=1.809) plants treated with EP3 ($\bar{x}=7.67$) and E2 ($\bar{x}=8.40$) treatments remained with significantly less settled psyllids when compared with Blank ($\bar{x}=13.40$) and Negative Control ($\bar{x}=12.40$) plants. At the 72 h observation, no significant differences were noted between all treatments ($p>0.05$). Both P2 and EP2 did not seem to affect the settling behavior of the psyllids throughout all the assay: although the number of observed psyllids was inferior in these plants than

the ones on the Blank and Negative Control, its difference was never statistically significant ($p > 0.05$) (Figure 18).

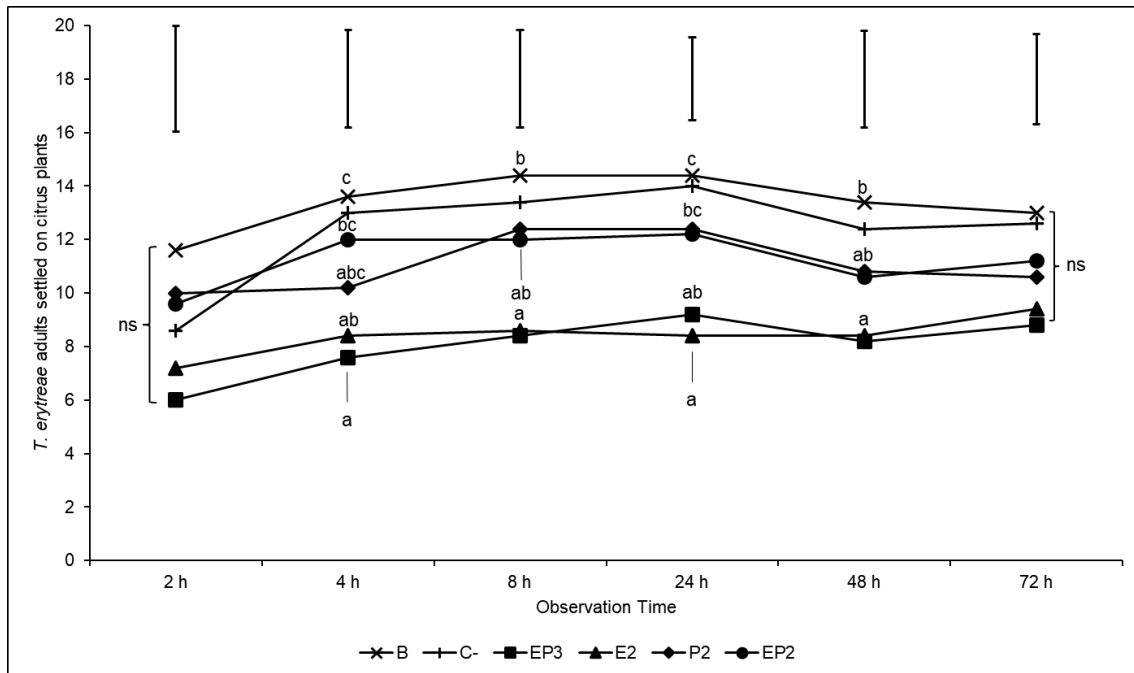


Figure 18 - Mean number of settled *T. erytreae* adults ($n=5$) through time. Error bars represent the least significant differences from Fisher's LSD post-hoc test, where data was analysed independently for each period of observation (2, 4, 8, 24, 48, and 72 hours). Different letters represent significant differences between treatments as per Fisher LSD ($p < 0.05$). "ns" signifies for no significant difference ($p > 0.05$).

For Oviposition, One-Way ANOVA showed the existence of significant differences between treatments in all of the observations: 2 h ($F(5,24)=7.042$; $p < 0.01$); 4 h ($F(5,24)=5.670$; $p < 0.01$); 8 h ($F(5,24)=11.586$; $p < 0.01$); 24 h ($F(5,24)=8.638$; $p < 0.01$); 48 h ($F(5,24)=4.765$; $p < 0.01$); 72 h ($F(5,24)=5.110$; $p < 0.01$).

Through the repeated measures one-way ANOVA, it was possible to verify that time influences the psyllids oviposition, Wilks' Lambda=0.032, $F(5,20)=119.889$, $p < 0.01$. Furthermore, there was also a significant difference when pairwise comparing each observation period ($p < 0.05$), with an increment in the proportion of shoots with eggs, suggesting that the proportion of shoots with eggs increased significantly as time went by.

Results from the observation of oviposition deterrence are in line with the ones observed on the settling behavior, except for the 2 h (LSD=9.428) observation where P2 ($\bar{x}=43.33$) treated plants already had shoots with eggs, significantly differing from all the other treatments ($\bar{x}=0.00$, for all treatments) ($p < 0.01$) (Figure 19).

After 4 h (LSD=14.784) however, and for the following observations, plants treated with EP3 ($\bar{x}_{4h}=0.00$; $\bar{x}_{8h}=0.00$; $\bar{x}_{24h}=35.00$; $\bar{x}_{48h}=76.67$; $\bar{x}_{72h}=93.33$) and E2 ($\bar{x}_{4h}=6.67$;

$\bar{x}_{8h}=38.33$; $\bar{x}_{24h}=61.67$; $\bar{x}_{48h}=71.67$; $\bar{x}_{72h}=71.67$) exhibited significantly less shoots with eggs when compared with the Blank ($\bar{x}_{4h}=60.00$; $\bar{x}_{8h}=73.33$; $\bar{x}_{24h}=100.00$; $\bar{x}_{48h}=100.00$; $\bar{x}_{72h}=100.00$) and Negative Controls ($\bar{x}_{4h}=47.33$; $\bar{x}_{8h}=78.67$; $\bar{x}_{24h}=96.00$; $\bar{x}_{48h}=96.00$; $\bar{x}_{72h}=100.00$) ($p<0.05$). Furthermore, up until the 8 h (LSD=12.147) observation, no eggs were observed on plants treated with the EP3. In contrast, Blank and Negative Control plants already had an average of over 70% of the shoots with eggs (Figure 19).

Between EP3 and E2 treatments, EP3 may seem like the most effective in deterring the egg posture has also evidenced by the 8 h and 24 h (LSD=11.537) observation, where this treatment had significantly less shoots with eggs ($p<0.05$). However, at the last 72 h (LSD=7.297) observation, plants treated with E2 had now a significantly less proportion of shoots with eggs, even when compared with EP3 ($p<0.05$) (Figure 19).

Although not as great as EP3 and E2, EP2 ($\bar{x}_{8h}=53.33$; $\bar{x}_{24h}=71.67$; $\bar{x}_{48h}=71.67$; $\bar{x}_{72h}=83.33$) also seems to have some effect on oviposition. This treatment showed a significantly lower proportion of shoots with eggs when compared with the Negative Control after 8 hours ($p<0.05$), and with both Negative and Blank Control in the following 24, 48 (LSD=9.117) and 72 h observations ($p<0.05$) (Figure 19).

Apart from the 2 h observation, P2 did not present any significant difference from the Blank and Negative Control plants in egg posture ($p>0.05$) (Figure 19).

Data on mortality were both not normally distributed and without homogeneity of variance, as such, parametric tests could not be performed to assess differences in mortality. Instead, H Kruskal-Wallis (KW) non-parametric test was used to compared medians. This test was performed to compare differences in the median number of dead insects, between the different treatments, and independently for each time.

The KW test evidenced that there were no differences in mortality between treatments throughout all of the assay: after 2 h (H(5)=3.944; $p=0.558$); 4 h (H(5)=7.472; $p=0.188$); 8 h (H(5)=5.893; $p=0.317$); 24 h (H(5)=5.213; $p=0.390$); 48 h (H(5)=4.702; $p=0.453$); 72 h (H(5)=2.338; $p=0.801$). Since the number of live adults may directly affect the number of settled psyllids and the amount of shoots with eggs, the cumulative mean number of dead insects may be useful for settling behavior and oviposition discussion.

By observing the cumulative mean number of dead adults (Table 7) it can be noted that at the end of the assay (72 h), which was the time with the highest dead count, the amount of dead adults was larger on plants treated with the E4 ($\bar{x}=2.0$) compound. On average, plants treated with this compound only differed by one more dead insect compared with the Blank ($\bar{x}=1.0$) and Negative Control ($\bar{x}=1.2$) groups. As for the other

treatments, EP2 ($\bar{x}=1.4$) was the treatment with the average 2nd largest number of dead insects, followed by EP3 ($\bar{x}=1.0$) and lastly P7 ($\bar{x}=0.8$).

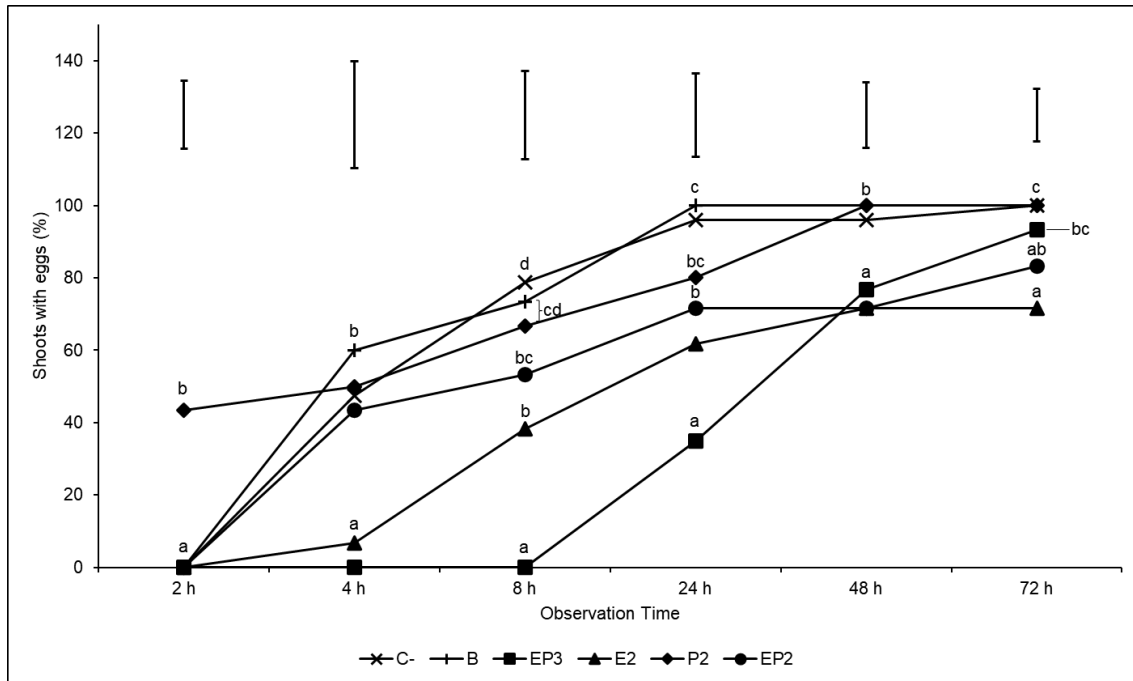


Figure 19 – Proportion of observed shoots with eggs (%) ($n=5$) through time. Error bars represent the least significant differences from Fisher's LSD post-hoc test, where data was analysed independently for each period of observation (2, 4, 8, 24, 48, and 72 hours). Different letters represent significant differences between treatments as per Fisher LSD ($p<0.05$).

Table 7 – Mean cumulative number of dead *T. erytreae* adults ($n=5$) registered through time (2, 4, 8, 24, 48 and 72 h) for each treatment.

Treatment	Dead <i>T. erytreae</i> Adults					
	2 h	4 h	8 h	24 h	48 h	72 h
C-	0.2	0.2	0.2	0.4	0.6	1.2
B	0.0	0.0	0.2	0.2	0.4	1.0
EP3	0.0	0.4	0.4	0.4	0.8	1.0
E4	0.2	1.2	1.2	1.2	1.8	2.0
P7	0.2	0.2	0.2	0.2	0.4	0.8
EP2	0.4	0.6	0.8	1.0	1.0	1.4

4.5. Ovicidal effect assay

To determine the ovicidal potential of the testing compounds, the Hatch Ratio (HR) was calculated and compared between each treatment as well as with the Blank and Negative Controls. Simultaneously, with the gathered data, the time for instar development was observed and treatments were similarly compared. Since two of the ANOVA assumptions were not met for both data analysis (population was not normally distributed and population's variances were not homogeneous), treatments were compared by applying a H Kruskal-Wallis non-parametric test.

No significant differences in the HR were observed between all treatments and controls ($H(4)=5.038$, $p=0.283$) at the 95% confidence level, indicating that none of the testing compounds had an ovicidal effect. It is still worth noting that the mean HR was the lowest in plants treated with P2 ($\bar{x}=85.83$), followed by E2 and EP2 ($\bar{x}=88.33$ for both). P2 also had the lowest observed HR at only 62.5% (Figure 20).

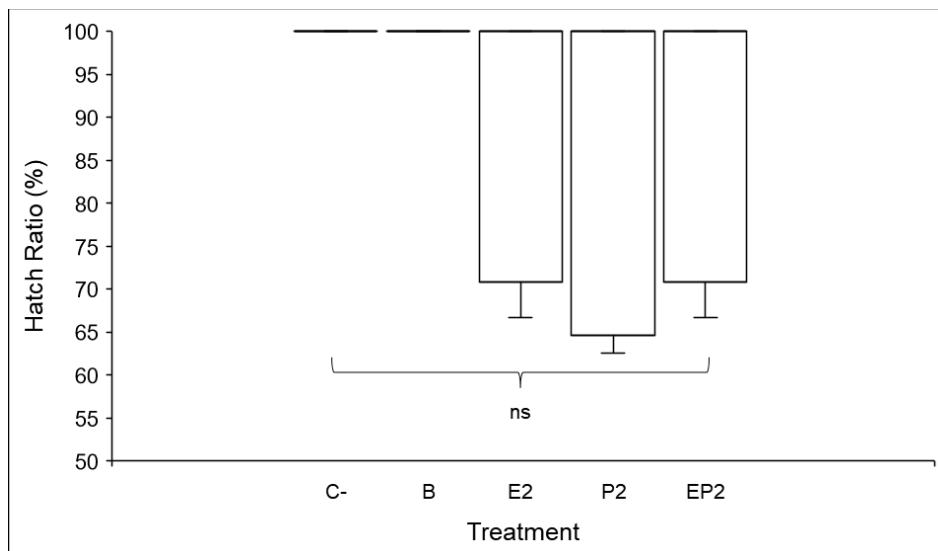


Figure 20 – Median observed Hatch Ratio of *T. erytreae* eggs ($n=5$), compared between each treatment. “ns” signifies for no significant difference as per Kruskal-Wallis test ($p>0.05$).

As for the nymphal stage registered in each observation, no significant differences were noted after 6 ($H(4)=4.800$, $p=0.308$), 7 ($H(4)=6.578$, $p=0.160$), 14 ($H(4)=7.474$, $p=0.113$) and 18 days ($H(4)=2.039$, $p=0.729$); but there were significant differences at the 5 ($H(4)=14.476$, $p<0.01$) and 10 days ($H(4)=10.164$, $p<0.05$) observations.

Both the 5 and 10 days observations shared the same differences, with all three tested treatments differing significantly from at least one of the controls. At the 5 days observation nymphs in plants treated with E2 ($\tilde{X}=0$), P2 ($\tilde{X}=0$) and EP2 ($\tilde{X}=0$) were at a significantly lower instar (no egg had still hatched) than in B ($\tilde{X}=1$) and C- ($\tilde{X}=1$) control

treatments ($p < 0.05$), where first instar nymphs already started to appear. After 10 days, nymphs in seedlings treated with E2 ($\bar{X}=2$), EP2 ($\bar{X}=2$) and P2 ($\bar{X}=2$) were significantly less developed than when treated with the C- ($\bar{X}=3$) control ($p < 0.05$). B control ($\bar{X}=3$) did not differ significantly from neither of the other treatments. In all observations, B and C- were never significantly different from each other and so did E2, P2 and EP2 whose differences were also never significant ($p > 0.05$) (Figure 21).

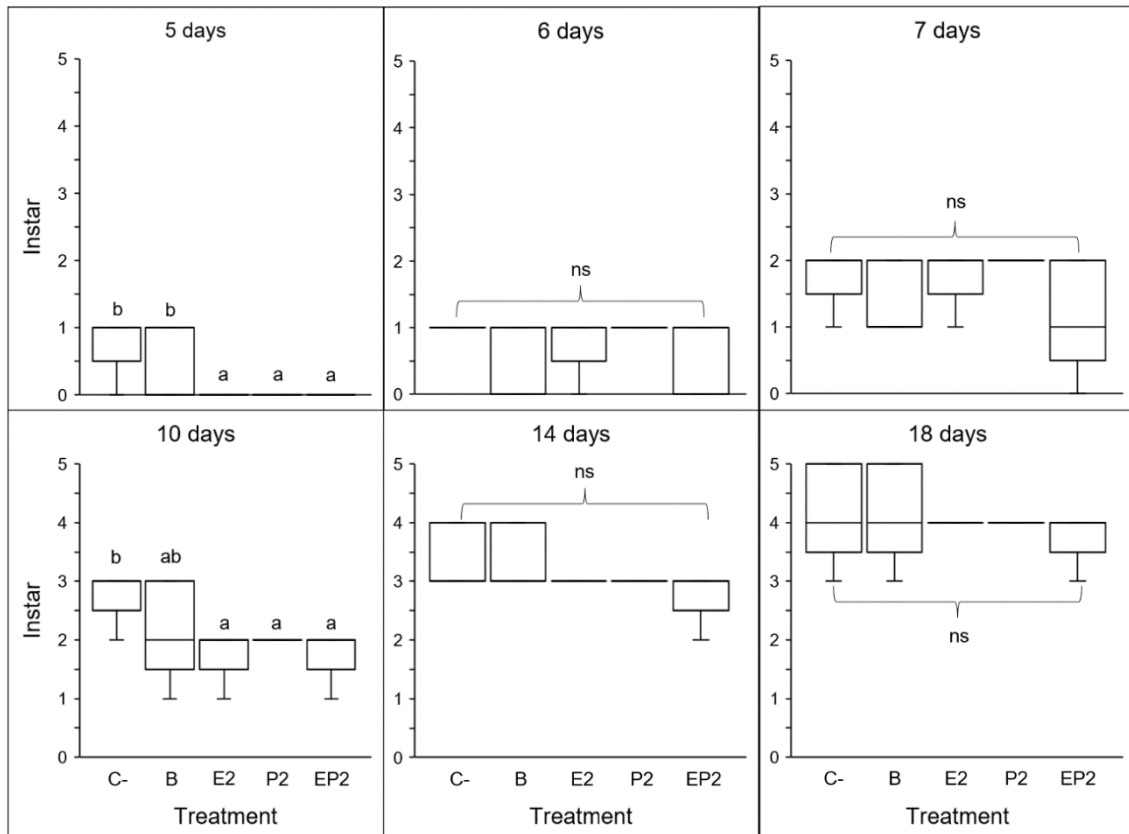


Figure 21 - Median observed *T. erytreae* instar ($n=5$) after 5, 6, 7, 10, 14 and 18 days, compared between each treatment. Different letters represent significant differences between treatments ($p < 0.05$) and “ns” signifies for no significant difference as per Kruskal-Wallis test ($p > 0.05$). Data was analysed independently for each observation time.

4.6. Semi-field assay

The solution that presented the most favorable results in the previous assays (EP3), and thus represented the highest potential for the practical application of the tested treatments, was tested on semi-field conditions. Furthermore, the spray application of the solution was divided in two different volumes of application, V1 and V2. The reduction of nymphs (Nymph Reduction (%)) in each tested plant was compared between each application volume, as well as the respective observed Surface Coverage Area (SCA).

Regarding the SCA, it was possible to note that the reduction in volume V1 (6.40 mL) to V2 (1.60 mL) resulted in a mean reduction of 50.10% (V1: \bar{x} =95.87, s =1.44; V2: \bar{x} =45.77, s =7.09) (Figure 22).

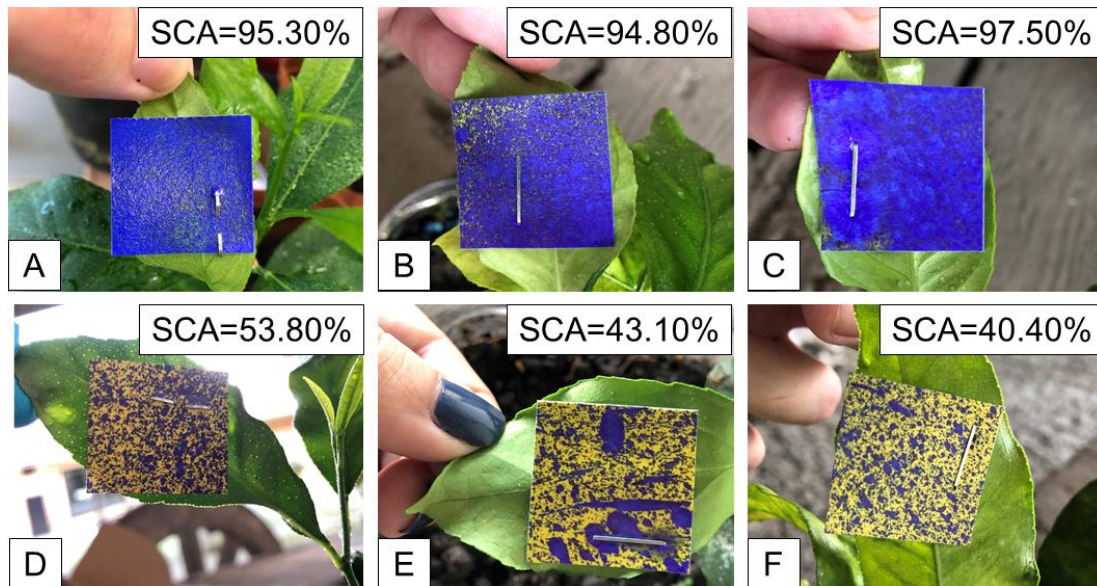


Figure 22 – Visual representation of the surface coverage area (SCA) in V1 replicates (A, B and C) and V2 replicates (D, E and F), with the respective indication of the SCA calculated value in the upper right of each frame.

As for the reduction in the number of nymphs in each *C. limon* plant, no significant differences were observed between the spray application of V1 and V2, both after 24 h ($F(1,4)=0.288$; $p=0.620$) and 48 h ($F(1,4)=0.841$; $p=0.411$), in the one-way ANOVA analysis (Figure 23).

Through the repeated measures one-way ANOVA, it was possible to verify that the reduction in the nymphs population (%) was significantly different between the 24 and 48 h observations Wilks' Lambda=0.207, $F(1,4)=15.314$, $p<0.05$, with an observed increment in the reduction of the nymphs population through time (Figure 23), evidencing that time increased the effect of the tested compound.

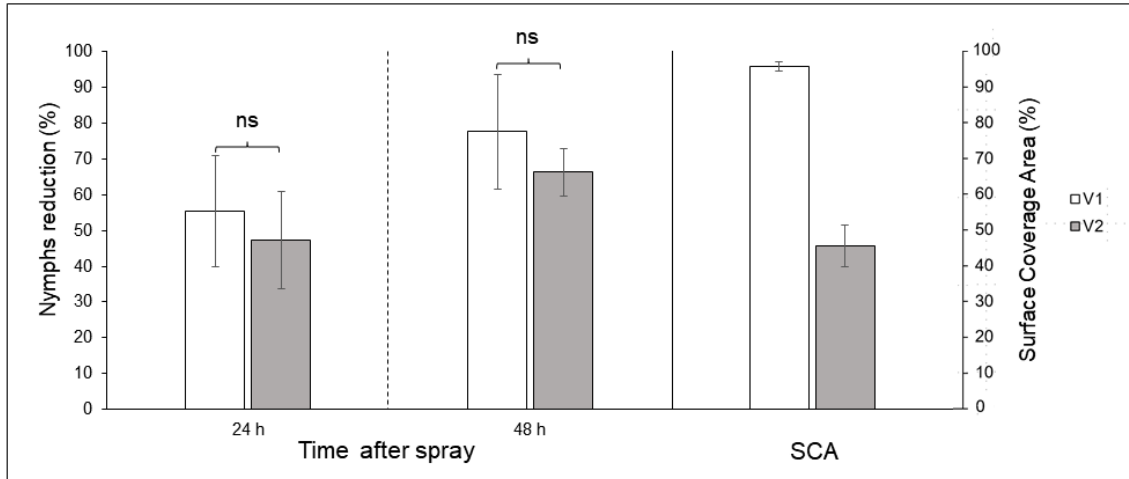


Figure 23 – Mean nymphal reduction (%) after 24 and 48 h of spray application with V1 (6.4 mL) and V2 (1.6 mL), as well as the observed Surface Coverage Area (SCA) respective to both V1 and V2 applications. Error bars represented by the standard deviation. “ns” indicates no significant differences as per One-Way ANOVA ($p>0.05$).

5. Discussion

This study assesses the efficacy of Eugenol and Pulegone, as well as their combination in the control of *T. erytreae*. Furthermore, the insecticidal application of these solutions was assessed by evaluating their topical toxicity, repellency and ovicidal effects as well as their phytotoxicity to *C. limon*, and their applicability in semi-field conditions, permitting a better view on the potential application of EOs and their active compounds as an alternative method to control *T. erytreae*.

5.1. Toxicity bioassay

In this study, toxicity was observed in *T. erytreae* nymphs for all tested treatments, although not all treatments expressed the same level of toxicity at the same concentration. The concentration at which each solution was applied directly influenced their toxicity, with higher mortality observed for concentrations above 1.60%. At a first glance, no significant differences were noted between all the treatments when applied at the same concentration, indicating that the effects on mortality of these solutions were equal. However, it was observed that applying Pulegone (either isolated or in combination with Eugenol) at double the concentration of Eugenol significantly increased the mortality, whereas no significant difference was noted in utilizing Eugenol at double the concentration of either Pulegone containing solutions. These considerations are strengthened by further evaluating the data in the Probit analysis, where Pulegone had lower estimates of both LD₅₀ and LD₉₀, after 24 h and 48 h. Pulegone usage as an insecticidal agent is well known and many are the reported effects of its direct application against Hemipterans such as *P. ficus* (Borau et al., 2020) and numerous other species of aphids (Ikbal & Pavela, 2019). Also, Eugenol has demonstrated insecticidal activity when applied by direct contact in Hemipterans such as *D. mangiferae* (Gaire et al., 2019; Ghafoor et al., 2019), *T. infestans* (Moretti et al., 2013), *A. craccivora* (Tewary et al., 2006) and even *C. chinensis* (Tian et al., 2015), a closely related psyllid.

The higher toxicity of Pulegone shown in these results are compatible with the ones observed on *M. pruinosa* nymphs, where *M. pulegium* EO was applied by direct contact and also exhibited higher toxicity than *S. aromaticum* (Kim et al., 2013). Contrarily, Lee et al. (1997) observed that while Pulegone and Eugenol have shown great toxicity against *T. urticae* adults at a concentration of 10000 ppm in a leaf dip bioassay (100% mortality after 24 h), at a lower concentration of 1000 ppm, only Eugenol showed some toxicity (60% mortality after 72 h). In the toxicity bioassay, the minimal concentration tested was

at 0.40%, corresponding to 4000 ppm. At this concentration both Pulegone and Eugenol were toxic to *T. erytreae* nymphs, however, the toxicity was not assessed to concentrations as low as 1000 ppm, which may explain the author's results. The observed differences may also be due to different methodology applied (topical application vs leaf dipping) as well as the fact that different species were tested and at different life cycle stages. As such, higher toxicity for Eugenol vs Pulegone cannot be ruled out for lower concentrations and further experiments on their toxicity at lower concentrations should be conducted.

The toxicity bioassay only accounted for toxicity of these solutions on *T. erytreae* nymphs, but not on adults, on which different levels of toxicity may be observed. The literature suggests that such differences exist, and adults can even be more susceptible than nymphs to these solutions, meaning that the observations from the topical toxicity on nymphs may serve as a reference for toxicity on adults. Such observations were noted by J.-R. Kim et al. (2015) when testing the individual compounds of cinnamon EOs. The author noted that Eugenol was the most toxic compound against adults of *M. pruinose* but not against its nymphs. Kim et al. (2013) also had similar observations: while *S. aromaticum* EO did not present any toxicity at the tested concentrations, *M. pulegium* EO exhibited great toxicity in *M. pruinose* when tested in a leaf-dipping assay on nymphs (100% mortality at 500 mg.L⁻¹) as well as when applied as a spray on adults at a lower concentration (100% mortality at 2.50% concentration). Since there was no direct contact assay performed on adults of *T. erytreae*, it can not be however stated with certainty that the differences in mortality between adults and nymphs of this species would perform in a similar fashion and further investigation should be conducted to evaluate the insecticidal effects of these solutions on *T. erytreae* adults.

Mixtures of two or more EOs active compounds often can exhibit synergistic, additive and/or antagonistic toxic effects in insects, opening new prospects in the search for more effective uses of bioactive substances (Borau et al., 2020). The enhancement of the insecticidal effects of these compounds when combined can be attributed to i) changes in the physical properties of the solution, thus affecting the cuticular penetration (e.g., solubility and surface tension) (Tak & Isman, 2015) and ii) the ability of the compounds to target different sites within the insect nervous system (Gaire, Scharf, et al., 2020) enabling, for instance, the hyperactivation of the insect and causing an increment in the uptake of the insecticide consumption (Reynoso et al., 2020; Reynoso et al., 2018)

Synergism between different EOs has been demonstrated with Eugenol in a 3-blend with α -terpineol and cinnamic alcohol (Enan, 2001). In a topical application assay on bed bugs, a tertiary 1:1:1 mixture of eugenol, carvacrol and thymol resulted in a synergistic

increase in their toxic and neuroinhibitory effects compared with the effects associated with its individual application (Gaire, Scharf, et al., 2020). Pulegone also has exhibited its potential usage together with Limonene on Hemipterans as shown by Borau et al. (2020) where the inclusion of Limonene in a mixture of Pulegone, diatomaceous earth and lecithin formulation increased the mortality rates of *P. ficus* after 24 h and 48 h of spray application.

In this study, the combination of Eugenol with Pulegone proved beneficial after both 24 h and 48 h of application, always presenting an additive effect, an indication of the compatibility between both compounds. Synergism in the combination of both compounds was also observed, but only after 24 h and only regarding the LD₉₀ evaluation. This variation in the interaction between different times may be however the result of already high mortalities observed in the 48 h period for Eugenol and Pulegone, at the tested concentrations. Furthermore, differences between the efficacy of the compounds were more evident at the 24 h observation, enabling a better view of eventual interactions between the compounds. It was also noted that, at 0.40 and 0.80% concentrations, the combination of both compounds was less toxic (although not significantly) than both Eugenol and Pulegone individually. Such lower mortality for their combination may be due to the lower concentrations of each compound used in the mixture (E+P at 0.40% had 0.20% of both Eugenol and Pulegone). Due to the different mechanisms of action of each compound, the respective dosages of each independent substance in the mixture might determine the increment in toxicity for higher concentrations, but not for lower concentrations, hence the observed synergism for LD₉₀ evaluations but not for LD₅₀.

As previously mentioned, the synergistic and additive interactions of these compounds were to be expected due to both compounds having different mechanisms of action. Toxicity of Pulegone seems to be related to the production of menthofuran, a highly toxic organic compound that appears to be yielded by cytochrome P450 through the oxidation of Pulegone. Although metabolization of Pulegone has only been sparsely studied in insects, its relation with the activity of P450 has been observed in larvae of *Spodoptera eridania* and *Spodoptera frugiperda* (Gunderson et al., 1986), adults of *Musca domestica* (Rossi et al., 2012) and *P. ficus* (Peschiutta et al., 2017). Additionally, Pulegone insecticidal effects may also be related to the inhibition of acetylcholinesterase, as demonstrated by Herrera et al. (2015) but other mechanisms may be involved due to a non-direct correlation between the toxicity of the tested oils and the registered percent inhibitory activity of acetylcholinesterase. Pulegone action as a feeding deterrent has also been documented when studying the compound in *Myzus persicae*. It was noted

that Pulegone deterred aphid probing and feeding at preingestional, ingestional and postingestional phases of feeding (Dancewicz et al., 2008).

Regarding the mechanisms behind Eugenol synergistic activities, hyperactivation has been reported in various insects exposed to Eugenol in lower dosages, causing an uptake in insecticide consumption. Nymphs of *T. infestans* picked up more insecticide when hyperactivated by Eugenol, becoming intoxicated faster, as reported by Reynoso et al. (2018). On the same insect, Eugenol has also shown an increase in velocity, moving time, and distance traveled by the nymphs, effects that may be associated with its apparent action on octopamine receptors as the same response was observed when octopamine itself was topically applied (Reynoso et al., 2020).

5.2. Phytotoxicity assay

The direct application of EOs as an insecticidal control agent may result in phytotoxic effects in the treated crops, as such, the evaluation of EOs efficacy should account for possible phytotoxicity. Phytotoxicity and the severity of the plant injury may be dependent on the concentration and dosages applied, plant species, and plant organs exposed during the application, the physiological state, and product formulation (Cloyd et al., 2009; Werrie et al., 2020). Phytotoxic activity of EOs is attributed to the activity of the various compounds within the oil and their interactions - often synergistic - including enzyme inhibition, chlorophyll degradation, electrolyte leakage and membrane damage (Kimbaris et al., 2017). The results of this study have shown the interactions between the treated *C. limon* plants with the applied treatments, which were shown to be both dose- and time-dependent. Phytotoxicity advanced in each consecutive observation, and the phytotoxic effects of all tested treatments were exacerbated with every increment in concentration and were concurrent with the observations of Cloyd et al. (2009) and Werrie et al. (2020), in their reviews on topic.

After 9 days of observations, every treatment tested was phytotoxic at concentrations $\geq 3.20\%$. Although the phytotoxic assay was performed on six- to eight-month-old *C. limon* potted plants, the observed symptomatology suggests that when utilizing these solutions at these concentrations, one should account the probable effects on the plant and thus avoid these dosages. Regardless, further investigation on full-grown grafted *C. limon* trees could be explored to better aid the decision-making process.

For the inferior tested concentrations, only Eugenol at 1.60% presented non-acceptable phytotoxicity according to the created Phytotoxicity Index. The effect of Eugenol on the

plants was always greater than Pulegone in all tested concentrations, a result accordant with Lee et al. (1997) where Eugenol presented greater toxicity than Pulegone on corn roots and leaves. The author referred to the complete absence of phytotoxic effects with the application of Pulegone which does not concurs with the presented results, however the maximum applied concentration by the author was 0.05%, a much lower concentration than the lowest used in this test (0.80%).

M. pulegium EO, and especially Pulegone, has also been shown to be safe for usage, with moderate to no observed phytotoxicity on tomato (*Solanum lycopersicum*), cucumber, *Phaseolus* sp. and eggplant seedlings (4 $\mu\text{L.L}^{-1}$ air and 1000 $\mu\text{L.L}^{-1}$ spray application for tomato) (Papadimitriou et al., 2019; Topuz et al., 2018; Zandi-Sohani, 2011); *Lolium perenne* and *Lactuca sativa* (Kimbaris et al., 2017; Santana et al., 2014); bean plants (20 $\mu\text{L.L}^{-1}$ air) (Zandi-Sohani & Ramezani, 2015); vine leaves (1200 $\mu\text{L.L}^{-1}$) (Borau et al., 2020); Pulegone has also shown to not affect *S. lycopersicum* germination at 0.4 mg.mL^{-1} (Kimbaris et al., 2017).

Similar to this study's observations, other reports of high phytotoxicity from Eugenol application have been published. Eugenol is known for its herbicidal activities and is regarded as highly phytotoxic. As an herbicide, clove oil is a contact, non-selective foliar herbicide that will only control above-ground, green vegetation. It causes rapid loss of cellular membrane integrity and does not translocate (Copping & Duke, 2007). Hall et al. (2018) reported extensive necrosis of mature citrus leaves as well as shoots of young plants when treated with concentrations over 5.00% of a commercial mixture of oils from orange, clove, cinnamon, eucalyptus and rosemary. Cloyd et al. (2009) also registered high phytotoxicity on *Gerbera jamesonii* and *Euphorbia pulcherrima* for several essential oil-based commercial products containing clove EO. *S. aromaticum* EO (Eugenol 84% v/v) at 5-10 % v/v concentrations was highly phytotoxic to *Chenopodium album*, *Ambrosia artemisiifolia* and *Sorghum halepense* where shoot death was observed within 1 h and 1 day after treatment.

The phytotoxicity assay was performed during the first 9 days after application, as such, the phytotoxic evaluation between all treatments should account only for this period as phytotoxicity may progress for a longer period. Regardless, the usage of the combination of both oils at a concentration of 1.60% presented very favourable results, with a significantly lower phytotoxicity than Eugenol at the same concentration, and at an acceptable level. Similarly to the effect of the combination of both oils in the topical application assay, there seems to be an additive effect in phytotoxicity for higher concentrations when both Eugenol and Pulegone are combined, whereas for lower concentrations, the combined phytotoxic effects of both compounds is lower than the

phytotoxic effects observed when compounds are applied separately. Again, this behaviour may be explained by different mechanisms of action of these oils in the plant tissues. The mechanisms of action of EOs on phytotoxicity are still yet not fully understood, however, Eugenol seems to affect the chlorophyll content, photosynthetic efficiency and cellular respiration by altering the mitotic activity, disorganizing the microtubule organization and altering the biosynthesis of cell wall whereas Pulegone acts as an uncoupler agent of mitochondrial respiration (Maffei et al., 2001; Mucciarelli et al., 2001; Tworkoski, 2002; Verdeguer et al., 2020). To better understand the implications of utilizing EOs for crop protection, further research must be conducted on understanding these EO-crop interactions. A better view of the specific sites of action of these plant-based compounds, as well as their effect on crop yield should be very useful in the search of valid alternatives for crop protection pesticides.

5.3. Repellency assay

The term “repellent” is used to describe a chemical that produces an oriented response from the insect away from the chemical source (Dethier et al., 1960). In this study, repellency on *T. erytreae* adults was evaluated by observing their settling and oviposition behavior when enclosed with *C. limon* seedlings previously sprayed only with the testing solutions that presented acceptable phytotoxicity. The correct evaluation of these effects requires the usage of merely non-phytotoxic treatments, as only then it is possible to objectively relate the behavioral responses of the psyllid with the tested solutions, as opposed to a possible repellence due to the plant’s poor health. Such constraints in observing behavioral responses were noted by Hall et al. (2018) when studying the repellent effects of commercial mosquito repellents and EOs on *D. citri*. The author noted that few psyllids colonized the seedlings, infestations were reduced and oviposition was strongly suppressed when the testing compounds were applied at 5-25% concentrations but, due to high phytotoxicity on the citrus seedlings, none of these effects could be attributed to the tested compounds.

In this study, the number of settled *T. erytreae* adults on *C. limon* was affected by the treatment applied, as well as the time after application. From all the treatments applied, only plants treated with the combination of both oils at 1.60% (EP3) and Eugenol at 0.80% (E2) presented a significantly lower number of settled psyllids when compared with the Blank Control. Contrarily, the application of Pulegone (P2) and the combination of both oils at 0.80% concentration (EP2) did not seem to affect the settling behavior of these psyllids, evidencing a stronger repellent activity by Eugenol vs Pulegone. Since

EP2 and EP3 showed significantly different results although utilizing the same compounds at the same proportion (1:1), and only Eugenol was repellent when tested separately, the differences in results between both combinations thus seem to be reliant on the concentration of Eugenol present in the mixture. In this case the compounds with 0.80% Eugenol (E2, EP3) affected the settling behavior, while the treatment with 0.40% Eugenol (EP3) did not. This hypothesis would be in agreement with the observations of Mann et al. (2012), where Eugenol at 0.50% concentration did not produce a behavioral response on *D. citri*. However, the possible influence of Pulegone in the mixture should also be accounted for. More so, the author did not test the influence of Eugenol at a higher concentration and the methodology applied was different (T olfactometer assay). As such, to correctly assess the potential of Eugenol as a *T. erytreae* repellent, further studies could be conducted by testing different concentrations of the compound, in small increments. Few studies have also been performed regarding the repellency of Pulegone and are generally more focused on its toxicity.

Eugenol repellent effects on insects are well known (Isman, 2000; Obeng-Ofori & Reichmuth, 1997), and many authors have also described its effects on hemipterans: Diaz-Montano and Trumble (2013) observed repellency for clove oil on the potato psyllid *Bactericera cockerelli* on a y-olfactometer assay when 10-1000 µL of the oil was applied either on a filter paper or a tomato leaf. The author further noted the residual effect of the compound, where it repelled between 77- 91% of *B. cockerelli* adults during a 20 day trial; Moretti et al. (2013) registered that *T. infestans* and *Rhodnius prolixus* nymphs were flushed out when sprayed with a aerosolized solution of Eugenol; and de Andrade et al. (2013) noted that *S. aromaticum* EO at 0.05% concentration reduced by 50% the number of *Aedes aegypti* nymphs present in leaf discs after 48 h, but at such concentration the compound only repelled 25% of adults.

As for Pulegone, even though in this study no effects were observed for this compound, repellency was observed by other authors in *R. padi* (Pascual-Villalobos et al., 2017) and *B. tabaci* although only at 10% concentration. Pulegone can act as an anti-feedant, as shown by Kimbaris et al. (2017) and Dancewicz et al. (2008), where it was noted the feeding inhibition of *M. pulegium* and Pulegone in *M. persicae*. These compounds influenced the feeding behavior of aphids by increasing the difficulty in finding and reaching the phloem, thus decreasing both the duration of phloem ingestion and the proportion of aphids with sustained sap ingestion and increasing the proportion of aphid salivation in phloem.

Only one comparison between the repellent effects of Eugenol and Pulegone in hemipterans was found. Zhang et al. (2014) conducted a study to assess and compare

the capability of certain EOs in blocking the action of bug attractants in *Halyomorpha halys* and therefore determine their repellency capabilities. The author noted that clove oil, and a tertiary mixture of clove, lemongrass and spearmint blocked the attraction to the stink bug attractant-baited traps. Furthermore, when compounds of these oils were tested individually, Eugenol and Pulegone presented great blocking effect to the attractants. Upon further analysis of the available data, Eugenol did seem to have a greater blocking effect than Pulegone, which was concurrent with this study's observations, however, in the authors assays this difference was not significant.

In accordance with the observed in *T. erytreae* in this study, Zhang et al. (2014) also did not observe significant differences between male and female adults on *H. halys*. The author noted that the sensorial perception of the volatiles seems to be similar for both Eugenol and Pulegone and both sexes. These observations occurred when testing the repellency of these compounds, were the author identified the presence and activity of these compounds in both males' and females' antenna through electroantennographic detection. Given that electrophysiological activity is an indicator that a compound may be behaviorally active, the activity of these compounds in the antenna may be indicative of their responsibility in the repellency of the EOs.

Since both compounds seem to present repellent effects according to the literature, whereas in this study, repellency was only noted for Eugenol, differences in the observed repellency were hypothesized to be related to the volatility of each compound. Regarding the effectiveness of a repellent, substances with higher vapor pressure and therefore volatile, can offer protection at lower concentrations in contrast with repellents with low vapor pressure (Debboun et al., 2006). On the other hand, less volatile repellents with subsequently lower evaporation rate are more likely to be more persistent and repel for a longer period. The effective results of the usage of a repellent are also dependent on humidity, temperature and wind factors influencing the evaporation rates and therefore its persistence (Moore & Debboun, 2007).

However, Eugenol has a much lower vapor pressure (0.022 mmHg at 25 °C) (Prates et al., 2019), than Pulegone (0.123 mmHg at 25 °C) (HSDB, 2021) which may explain a possible difference in persistence but not the observed higher repellency on Eugenol. As such, the differences may be then explained by the different mechanisms and sites of action of each compound i.e. Eugenol action in the octopamine receptors induces its hyperactivity (Reynoso et al., 2020) whereas Pulegone induces the formation of menthofuran, a highly toxic compound, through its oxidation on cytochrome P450 (Gunderson et al., 1986; Peschiutta et al., 2017; Rossi et al., 2012).

Regarding the persistence of the compounds, in this assay it was noted at the 24 h observation that *T. erytreae* adults were beginning to get caught up in the recipients' vertices and in the fabric-tulle covering at the EP3 and E2 treated enclosures, presumably due to a high repellency. Since after 24 h it was not possible to observe with confidence the time at which the psyllids returned to the plant, it was not feasible to assess if the treatments started to lose their effect after this period and thus determine their persistence.

Similar to the observed settling behavior, for oviposition, both time and treatment affected the oviposition. The observed results are in line with the observed settling behavior, where plants treated with Eugenol-containing solutions (E2, EP2, EP3) were the only ones that also had a significantly lower proportion of shoots with eggs vs shoots with no eggs when compared with the Blank Control. Such similarity was to be expected since the deterrence of oviposition is also a consequence of the psyllids avoiding the plant. However, it was noted that EP2 treated plants also had a significantly lower proportion of shoots with eggs than the Blank Control after 8 h whereas the same plants did not show significant differences in the number of settled adults with Blank Control, for the same period. Since Pulegone in this assay also did not demonstrate significant differences with the Blank Control, Eugenol may have some effect on oviposition other than keeping the psyllids from settling.

EOs volatiles may function as oviposition deterrents, even in Hemipterans (Koschier & Sedy, 2003; Pascual-Villalobos & Ballesta-Acosta, 2003; Yang et al., 2010), however for the compounds tested in this study, literature is still scarce on the subject. Eugenol did not act as an oviposition deterrent on *B. tabaci* but the applied methodology was significantly different (fumigant assay) and the concentrations of Eugenol applied was rather low, with a maximum of 1 $\mu\text{L}\cdot\text{L}^{-1}$ air (de Carvalho Ribeiro et al., 2010). On *T. urticae*, Pulegone had an oviposition deterrent activity but again as a fumigant application (Mozaffari et al., 2013).

Since the available literature seems to suggest that Eugenol does not affect oviposition, but Pulegone does, and thus contraries the results, the observed oviposition deterrence may be due to the lower number of settled adults in the plants. Nonetheless, the effects of these compounds on oviposition should be better assessed, with a larger range of concentrations while trying also to separate the effects of compound volatiles in settlement behavior with the effects of the same compounds in oviposition.

On this study assay, the effect of the testing solutions was also tested for their residual effect on mortality. No significant differences in mortality were also noted between all

treatments and Blank Control, and since the number of dead psyllids only accounted for a mean maximum of two psyllids, the cause for the observed mortality may simply be due to natural causes. Additionally, since the mean maximum difference of dead insects between treated and control plants only amounts to one psyllid, mortality seemed to affect neither the settling nor the oviposition behavior.

5.4. Ovicidal assay

The biocidal effects of EOs also extend to their efficacy in decreasing egg viability. From the available literature, both Eugenol and Pulegone seem to have some effect on egg viability in hemipterans, as described by Dias et al. (2019) when applying Eugenol at 1% concentration or higher, over 90% mortality on *Mahanarva spectabilis* eggs was observed; Mozaffari et al. (2013), Topuz et al. (2018) and Pavela et al. (2016) also observed a high toxicity of *M. pulegium* EO in *T. urticae* eggs. In fact, the ovicidal effects of EOs are often linked to the presence in the oils of oxygenated monoterpenes such as Pulegone (Pavela et al., 2016).

In this study however, neither Eugenol, Pulegone nor the combination of both compounds affected the Hatch Ratio (HR), indicating that none of the testing solutions had an ovicidal effect at the tested concentration. Nonetheless, the concentrations tested were only at 0.80%, as such ovicidal effects of these solutions at higher concentrations cannot be ruled out. Pulegone did seem to be the compound with the most potential as an ovicidal agent since it had the lowest observed HR, however it was not significantly different from the Negative Control.

The nymphal stage was also registered in each observation for 18 days. At the first observation, eggs treated with the Blank and Control solutions were already hatching and the presence of N1 instar nymphs was observed, whereas eggs treated with either Eugenol, Pulegone, or the combination of both compounds still did not hatch. This delay in the development of eggs and nymphs was consistent throughout the rest of the assay but significant differences between the development stage of nymphs in the Blank/Negative Control and the oil-treated plants were only again noted at the 10th-day observation. From this study observations, it seems that Eugenol, Pulegone and the combination of both compounds retarded the development of the eggs, which resulted in the observed differences in nymphal development, and no difference was noted between the compounds.

Even though the results of this study imply that the testing solutions may have some effect on the development of the eggs, the methodology applied may not have been optimal to observe the differences between treatments. To observe both the ovicidal and egg/nymphal development effect of the solutions, it would be necessary to quantify the initial number of eggs in each replicate and observe their individual development through time. This would however require the usage of a binocular lens or equipment with similar enlargement capabilities, which on the other hand could be incompatible with the observation in-vivo in the plants.

To account for egg mortality Dias et al. (2019) displayed groups of 15 *M. spectabilis* eggs in a petri dish for further application of a fixed volume of each testing solution per egg. Such methodology however would be difficult to replicate for *T. erytreae*, since its eggs protrude inside the leaf's tissue through its egg stalk. This egg stalk not only serves as an anchor point, keeping the egg attached in the leaf, but may also have a water-absorbing function (Moran & Blowers, 1967). As such, when detached from the leaf or when the leaf is cut-off and dries, eggs shrivel and die (Van der Merwe, 1923). One way to avoid this issue could be by utilizing the same methodology used in the topical application assay, where leaf's turgescence was kept by inserting the leaves petiole inside agar solution, nonetheless, it was noted that many times eggs were in shoots whose leaves petioles were still too short to be securely inserted inside the agar solution. For a non-invasive approach, a camera with a low enough focal length should be utilized, which would permit the image recording of the testing subjects in the plant and thus assess their development through time.

5.5. Semi-field assay

In this work, the practical application of the Eugenol and Pulegone mixture at 1.60% concentration was evaluated via spray application on six- to-eight-month-old nymph infested *C. limon* plants, in semi-field conditions. The results have shown that a decrease in application volume by four-fold resulted in a reduction in the spray coverage area (SCA), when compared to the typical until- runoff-application. Many are the intricacies affecting the effectiveness of a spray application in relation to the properties of the applied substance. From all the aspects influencing the quality of the spray, the droplet diameter and the coverage density of the droplets on the target seem to be the most important factors (Costa et al., 2017). Other properties such as the surface tension and the contact angle of the constituents of the spray may also influence the interaction of the spray solution with target surface, interfering with wind drag and evaporation potential

and the ability of the active compound in spreading over the surface (Almeida et al., 2020). However, the reduction in applied volume did not translate in a significant decrease in the number of nymphs present in the plants, both after 24 and 48 h of spray application, meaning that the typical application until runoff may not be mandatory to control the nymphal populations of *T. erytreae* utilizing these compounds. Similarly, Tian et al. (2015) also didn't observe significant differences in the reduction of *C. chinensis* nymphal populations when applying increasingly lower dosages of clove EO in pear orchards.

Furthermore, these semi-field results are compatible with the ones observed in the toxicity bioassay, where high mortality rates were observed for the same treatment. However, in the semi-field assay, due to the applied methodology, the observation of reduction in nymphal populations may not be directly related to their mortality, but rather other effects that may be in place, such as a nymphal flush out effect, especially in the lower dosages, as described by Moretti et al. (2017).

The further determination of these compounds effectiveness in field conditions was unfortunately not possible due to the unforeseen incidentals. An observation of the effects of both a Negative and Positive Control could aid in the better interpretation of the results. Furthermore, the additional test of a wider array of volumes, different droplets sizes and varied SCAs would help in determining the best application practices of these compounds for the control of *T. erytreae*. This would also allow for a better understanding of the reasons behind the reduction of nymphs in the plant not significantly following the differences in applied dosages. Since the nymphal development produces galls that result in leaf curling, leaves by themselves may be serving as a physical barrier, protecting the nymphs from the action of the sprayed solutions, thus avoiding the increment in effectiveness after a certain volume is applied. As such, for a better understanding of the underlying factors behind these observed results, further testing in field conditions should be ensued.

5.6. Final considerations and future applications

Following this series of assays, the combination of Eugenol and Pulegone at a concentration of 1.60% seems to be the best option for implementation as an alternative and viable insecticidal agent. This combination was not only significantly toxic to *T. erytreae* nymphs and repellent to its adults, but also presented a very favorable phytotoxicity, significantly inferior to the other tested compounds at the same concentration. Nonetheless, the effects of this combination, and its usage in an insecticide formulation, could be further enhanced.

In this study, the proportion of both Eugenol and Pulegone used in the mixture was 1:1 which did present good results but still may not be ideal. Since Pulegone was shown to have higher nymphal toxicity than Eugenol, while also presenting a more favorable phytotoxicity, proportions could be adjusted in order to include a higher amount of this compound in the mixture, all while keeping the demonstrated benefits of having a combination of these compounds. The inclusion of other EOs in the mixture, such as Dillapiol which has shown a great toxicity on *D. citri* nymphs and adults (Volpe et al., 2016), with different mechanisms of action could also be another sustainable approach and, as such, further investigation could assess possible compatible compounds in a tertiary mixture.

In the case of EOs, which are a mixture of highly volatile compounds, the usage of controlled release systems may help on protecting the compounds from environmental factors that degrade them, thus extending and maintain its effects over a longer period of time (Scher, 1999; Tarelli et al., 2009). Atomizer technologies such as *ISO-Mate*[®] and *CheckMate Puffer*[®] CM could be explored to enhance the repellent effects while decreasing phytotoxicity (Hall et al., 2018). Slow-release formulations such as encapsulation in a clay nanosheet like described by Mitter et al. (2017) could also result in extended residual repellency while circumventing phytotoxicity, as volatile compounds may still be released through pores present in the sheet while physically separating these same compounds from the sprayed citrus leaves. Encapsulation of *M. pulegium* EO on commercial baker's yeast prolonged its insecticidal activity by 3 days against *M. persicae* (Kavetsou et al., 2019). Higher repellent activity was registered when nano-emulsifying EOs, the smaller the particle, the higher the activity (Pascual-Villalobos et al., 2017). Since kaolin application has also been shown to reduce *D. citri* infestation and dispersal in flushing citrus orchards (Miranda et al., 2021), its usage as an EO microencapsulation clay also presents great potential and could be explored. Diatomaceous earth is a white, highly porous, powdery amorphous silica obtained from deposits of diatomite - fossilized sedimentary layers of tiny phytoplankton called diatoms (Quarles, 1992) and is widely known as an inorganic product that can facilitate the penetration of active compounds into the insect due to its ability to absorb epicuticular lipids, thus abrading the waxy layer that covers the cuticle of insects (Subramanyam & Hagstrum, 2012). The activity of diatomaceous earth is dependent on the insect in which it is applied due to differences in insects' physiology and anatomy but generally those with large surface area to volume ratios (often smaller insects) are more susceptible (Quarles, 1992) thus, its joint action with EOs could also be explored in *T. erytreae*.

Properties of the insecticide solutions based on EOs can also be modified by utilizing adjuvants in the formulations such as *Tween*[®] (which in this study's preliminary assays has shown to be toxic on *T. erytreae* nymphs) and soy lecithin. Soy lecithin is a good organic surfactant, even used in foods due to its null toxicity (Čilek et al., 2006) and has emulsifying, wetting and dispersing properties (Nyankson et al., 2015; Pascual-Villalobos et al., 2017). These adjuvants may even enhance insecticidal properties of the oils whilst reducing phytotoxicity when directly applied on crops, as demonstrated by Borau et al. (2020); it was also noted by the author that when using soy lecithin as an emulsifier, greater mortality was achieved, perhaps due to better mixture homogenization, greater stability and/or greater wettability with soy lecithin, thus improving the physiochemical properties of the spray. Pascual-Villalobos et al. (2017) also remarked that the usage of lecithin or lecithin with glycerol produced more stable formulations without affecting the activity of the tested compounds.

When combining the usage of insecticides and natural enemies for biological control in Integrated Pest Management (IPM) practices, one should also account for the possible incompatibility of these two control methods as, unfortunately, predatory arthropods are often more susceptible to commonly used insecticides than phytophagous ones (Ahn et al., 2001; Kim et al., 1999; Yi et al., 2006). When testing the fumigant toxicity of Basil (*Ocimum basilicum*) EO and its active compounds, against both *Thrips palmi* and one of its beneficial predators, it was observed that Pulegone induced stronger mortality on *T. palmi* than the synthetic insecticide dichlorvos, while at the same time being more selective towards the pest as shown by the lower mortality on *Orius strigicollis* – Pulegone was up to five times more toxic to *T. palmi* adults than to one of its recommended biological control agent *O. strigicollis*, hence exhibiting great selectivity (K. H. Kim et al., 2015). The same observations were reported by Yi et al. (2006), where the author observed that *O. strigicollis* was over 20 times less susceptible than *T. palmi* to *M. pulegium* EO.

T. dryi is one of the most effective parasitoids of *T. erytreae* and is already being used in the Iberian Peninsula to control *T. erytreae* expansion. As such, great care must be taken when developing new insecticidal control agents for *T. erytreae* since the selectivity of such compounds towards *T. dryi* is imperative. Dionisio et al. (2021) already tackled the effects of pine oil as an adjuvant to several insecticides. In the author's work he noted that the most effective insecticide for *T. erytreae* was cyantraniliprole, which also did not have a negative effect on *T. dryi*. Furthermore, the author evidenced that combining pine oil with this insecticide not only increased the mortality on *T. erytreae*, while also reduced the effect of the insecticide on *T. dryi*. The assessment of the toxicity

of Eugenol and Pulegone on *T. dryi* should also then be explored following this study in order to effectively obtain a viable alternative control solution. Additionally, these compounds should also be evaluated in the combination with other synthetic insecticides, mainly cyantraniliprole, due to its observed potential.

The prospective usage of EOs together with synthetic insecticides lays on its potentiator effects when combined, enabling a lower application dosage of insecticide while keeping the control viable. Such experiments have been gaining popularity but remain rather scarce, nevertheless, potentiator and/or synergistic and/or antagonistic effects of EOs have been reported when combined with insecticides (Abbassy et al., 2009; Faraone et al., 2015; Mesbah et al., 2006). Commercial insecticides with blends of EOs containing Eugenol are readily available such as *Bed Bug Patrol*[®], *Rest Assured*[®] and *Stop Bugging Me*[®] albeit with low concentrations of the compound (0.30%, 0.30% and 5% respectively) (Singh et al., 2014). However, a cautious approach should be taken since some combinations may produce an antagonistic effect, indicating not only that secondary constituents of the oils play a role in the synergism power, but also that synergistic activity is dependent on the combined insecticide (Faraone et al., 2015).

The combination of both Eugenol and Pulegone at 1.60% concentration was demonstrated in this work to be the best approach in utilizing these compounds for the control of *T. erytreae*. Nonetheless, by i) adjusting the proportions in the mixture; ii) adding other bioactive EOs in the mixture; iii) exploring the potential usage of clay microencapsulation; iv) using adjuvant emulsifiers; v) combining these compounds with insecticides and vi) assess their effects on *T. erytreae* natural enemies, the practical application of this mixture could be further enhanced, thus allowing the formulation of Eugenol+Pulegone based products with greater insecticidal capability, while maintaining acceptable levels of phytotoxicity and enabling their prospective usage in IPM agricultural environments.

6. Conclusion

This study demonstrated the effects of selected EOs active compounds and their combination on *T. erytreae*, thus evidencing their potential usage as effective alternative control agents. Furthermore, the applicability of these solutions on *C. limon* was evaluated allowing a better insight on their effects on citrus crops, supporting an eventual integration of these compounds for citrus orchards protection solutions. In a series of laboratory and semi-field assays, Eugenol and Pulegone nymphal toxicity, repellency and ovicidal effects, as well as their phytotoxicity on *C. limon* plants was assessed. From the components tested separately, Pulegone had the highest effect as a nymphal insecticide, whereas Eugenol has shown the best repellent potential but also demonstrated to be the compound exhibiting the worst phytotoxic effects. At a concentration that did not pose significant phytotoxicity, the combination of both active compounds exhibited the highest nymphal mortality while also keeping the repellent effect.

The beneficial integration of both Eugenol and Pulegone in a mixture was shown to be the best approach for utilizing these compounds, but care in their concentrations must be taken, given the possible effects on phytotoxicity. This mixture has shown to be toxic on *T. erytraeae* nymphs, as well as affect the adult's settling behaviour, all while presenting very favourable phytotoxicity on *C. limon*. The mixture also seemed to present some oviposition deterrence effect, but the results were not conclusive due to the methodology applied and further investigation of the oviposition effects of these solutions should be performed. In a practical application in semi-field conditions, at a 1.60% concentration, this mixture was able to reduce the nymphal populations of *T. erytreae* in *C. limon* plants by 70%, showing its potential as an alternative control method.

The possible incorporation of these compounds for the control of *T. erytreae* shows promise, but there still is room for improvement through their combination and integration with other technologies, agricultural practices, and other botanical and synthetic insecticides. To better understand the potential integration of these compounds in IPM practices, a better insight of the interactions between these compounds and *T. erytreae* natural enemies, especially *T. dryi*, should also be assessed. Nevertheless, this study expands the knowledge on the potential effects of EOs on *T. erytreae* and their applicability in citrus crops, thus opening new prospects for the development of more sustainable approaches for this pest control.

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